METABOLIC ALTERATIONS IN AIRWAY EPITHELIUM IN ALLERGIC ASTHMA

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Abstract

Allergic asthma is a chronic inflammatory disease of the lung. It is thought to arise as a result of aberrant type-2 immune responses to environmental allergens in susceptible individuals. Among the allergens, sensitization to house dust mite (HDM) is one of the major risk factors for the onset of asthma. However, the mechanisms driving the initiation of such inappropriate type-2 immune responses following allergen exposure remain unclear. Recent studies have shown that the release of the cytokine interleukin 33 (IL-33) from airway epithelial cells following allergen recognition by pattern recognition receptors (PRRs) expressed on these cells dictates the nature and magnitude of the immune responses. However, the allergen-induced signals regulating the release of IL-33 remain to be fully established. Based on recent evidence that asthma is associated with increased glycolysis activity in the lungs, we hypothesized that HDM exposure induces increased glycolysis in airway epithelial cells, facilitating the secretion of IL-33 and allergic airway responses. We report for the first time that HDM induces immediate increases in glycolytic flux in airway epithelial cells that is associated with the early release of IL-33. Mechanistically, we confirm that HDM-induced IL-33 release is mediated by cytoskeletal rearrangement, which results in rapid deprivation of intracellular ATP. Such energy deprivation leads to activation of the homeostasis regulator AMP-activated protein kinase (AMPK), resulting in increased glycolysis and production of glycolytic ATP that in turn, facilitates the translocation of IL-33 to the extracellular milieu. Further analyses reveal that blockade of glycolysis *in vivo*

abrogates HDM-induced allergic airway responses likely via inhibition of IL-33 release, which in turns inhibits type-2 cytokine IL-13 production from its potent early innate source, type-2 innate lymphoid cells (ILC2s).

Collectively, these results suggest that allergic asthma may arise due to allergen-driven changes in airway epithelial cell metabolism that facilitates early, immediate release of IL-33 and the initiation of type-2 inflammation through recruitment and activation of ILC2s. Prevention of allergen-driven metabolic changes in the airway epithelium may provide a means to mitigate the initiation of type-2 immune response-mediated inflammation and the onset of asthma, offering a promising new therapeutic avenue for combating this disease.

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Chapter 1: Introduction and background

1.1 Asthma

1.1.1 Disease characteristics

1.1.1.1 Definition

The Global Initiative for Asthma (GINA) characterizes asthma as "chronic airway inflammation and a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness, and cough that vary over time and in intensity, together with variable expiratory airflow limitation" (GINA, 2015) [1]. Clinically, this disease is commonly defined by the presence of reversible airflow obstruction, airway hyperresponsiveness (AHR), and airway inflammation [2]. It is believed that the pathological outcomes of asthma arise as a result of underlying inflammation of the airways caused by inappropriate immune responses to common allergenic stimuli in genetically susceptible individuals [3]. Asthma is also recognized as a complex disease varying in clinical manifestation, severity, the degree of reversibility, and the responsiveness to current therapies [4].

1.1.1.2 Prevalence and public health impact

Asthma is a common global public health problem. The World Health Organization (WHO) estimated that, conservatively as many as 300 million people of all ages and ethnic backgrounds were suffering from asthma by the year 2006 [5]. In recent decades, the prevalence of asthma in both children and adults has greatly

increased in parallel with an increase in atopic sensitization [6]. It is estimated that an additional 100 million people will be diagnosed with asthma by 2025 [6].

Asthma prevalence in the United States (U.S.A.) has also rapidly increased over the last few decades. According to the Centers for Disease Control and Prevention (CDC), asthma prevalence increased from 3.1% in 1980 to 5.5% in 1996 and 7.3% in 2001 (20.3 million persons diagnosed). However, it increased to 8.2% (24.6 million persons diagnosed) in 2009, representing an increase of 12.3% in those eight years alone. Among those individuals affected by this disease, the prevalence among children (aged under 18 years) was highest compared to other age groups and reached 9.6% in 2009 [7]. A more recent report from the CDC noted that 13% of adults aged 18 and over were diagnosed with asthma in 2012 [8]. Significant health disparities associated with socioeconomic and racial status were observed, as women, minorities, and those unemployed or living below the poverty line were more affected [7, 8].

Asthma has caused great economic burden to the society as well. It resulted in a total indirect cost (e.g., productivity loss) of $8.2 billion and a total direct cost of $7 billion (e.g., hospital admission) in 1997 in the US [9]. A recent CDC report suggested that the financial burden of disease reached $56 billion in 2009, with an annual increase of $1.5 billion [10]. Thus, asthma remains a major public health concern, resulting in an urgent need for the development of more effective prevention and control approaches.

1.1.1.3 Determinants of asthma

(1) Genetic susceptibility

Although asthma is known as a heterogeneous disorder of unknown etiology [3], susceptibility to asthma is thought to result from the interaction between intrinsic genetic predisposition and extrinsic environmental factors [11]. Children whose parents have atopic diseases are more likely to develop allergic asthma [12, 13], indicating a heritable component of the disease. This hypothesis is further supported by multiple twin studies showing that there is a significant concordance in monozygotic twins as compared with dizygotic twins [14-16].

There have been numerous studies focusing on identifying genetic risk factors for allergic asthma utilizing genome-wide approaches and candidate gene association studies [17]. In early genome-wide linkage studies of asthma, more than 20 distinct chromosomal regions were implicated in susceptibility to a diagnosis of asthma or the related traits (See review in [17]). Collectively, these previous studies suggested that certain chromosomal regions such as 2p (cytotoxic T-lymphocyte-associated protein 4 (CTLA4)), 5q23-31 (cytokine gene cluster, cluster of differentiation 14 (CD14), B-adrenergic receptor), 6p24-21 (the major histocompatibility complex (MHC)), 11q13-21 (near the B chain of the high affinity receptor for IgE), 12q21-24 (stem cell factor (SCF), interferon-γ, and signal transducer and activator of transcription-6 (Stat6), etc.) might harbor asthma susceptibility genes [17]. The application of advanced genome-wide association studies (GWAS) has allowed more comprehensive discoveries of asthma susceptibility genes. For example, a large scale GWAS in Europeans (GABRIEL Study) found that SNPs in IL1RL1/IL18R1, HLA-DQ, IL33, SMAD3 and IL2RB were

significantly associated with allergic asthma [18]. Another recent meta-analysis of GWAS datasets (EVE Consortium) showed that four loci within/near IL1RL1, TSLP and IL33, and one locus within PYHIN1 were significantly associated with allergic asthma across various ethnic populations [19].

In addition to the genome-wide analyses, candidate gene studies account for the majority of genetic identifications for asthma determinants. These studies test whether the frequencies of particular specific genetic variants are more prevalent in asthmatic patients (cases) than in healthy individuals (controls) [17]. These genetic variants were mainly selected based on prior hypothesis-driven studies that sought to identify an association between variants in pathways influencing allergic asthma or relevant phenotypes [20]. It was concluded that most allergic asthma risk genes were involved in innate immunity and antigen presentation, Th2 polarization, and epithelial cell function (see review in [20]).

(2) Allergens.

The generational interval necessary to introduce a genetic predisposition to allergic asthma, along with lack of 100% concordance in twin studies suggest that the recent rise in asthma prevelance cannot be exaplined by genetics alone, and that, environmental factors play significant roles in the development of allergic asthma as well. In fact, environmental factors such as allergens [21], pollutants [22] and viruses [23] have been reported to play pivotal roles in asthma and allergy. Among these factors, allergic asthma is more likely induced by allergens [24], and it is believed that exposure of genetically predisposed individuals to environmental allergens increases the rate of asthmatic and allergic exacerbations [25].

Allergens are derived from a variety of sources including plants, insects, fungi, mold, and mammals [26]. Among different allergens, aeroallergens derived from the house dust mite (HDM) are known to be powerful inducers of allergic responses in children [27]. Epidemiological studies have shown that at least 60% of asthmatics are sensitized to HDM [28], and this number is closer to 80% among children [29]. Further prospective cohort population studies [30] and data derived from experimental murine models [31] have confirmed that HDM exposure is sufficient to induce the onset of allergic asthma. Although it is widely accepted that environmental exposures lead to dysregulated immune responses and subsequent pathological changes in the host, the underlying mechanisms by which environmental factors such as allergens induce allergic airway responses are not well understood [32].

1.1.2 Pathophysiology

Patients with allergic asthma (i.e., the most common form of asthma) generally have higher levels of circulating IgE antibody and are skin test positive to common allergens. Such IgE antibody is produced via a tightly regulated, complex network of cellular and molecular events necessary for the development of asthma pathophysiological manifestations. Specifically, it was reported that during an asthma attack, the presence of inflammatory cells in the bronchoalveolar lavage (BAL), sputum and lung biopsies is normally observed [33-35]. Thus, it is believed that inflammation is probably one major underlying cause of allergic asthma. Further understanding of detailed pathophysiology leads to the proposed scenario

that in susceptible individuals, airway inflammation arises due to inappropriate immune responses to common aeroallergens following recognition and presentation of antigens by airway epithelium and antigen presenting cells (APC) to CD4+ T cells in the draining lymph nodes. These allergen specific T cells polarize to a T helper (Th) 2 phenotype and produce the Th2 associated cytokines IL-4, IL-5 and IL-13. Collectively, these cytokines activate a series of events leading to IgE antibody production as well as recruitment and activation of a variety of downstream effector cells such as eosinophils, mast cells, and basophils [36]. Upon re-exposure to sensitizing allergens, sensitized individuals develop both early and late phase responses [37]. During an early phase response, allergens crosslink with IgE and induce mediator release from mast cells that result in bronchoconstriction and edema [38], while eosinophils and lymphocytes dominate the late phase allergic responses [39]. Continued chronic exposure to allergens results in the migration of inflammatory cells into local tissues, and pathological structural and functional changes such as fibrosis, goblet cell hyperplasia, excessive mucus production, as well as smooth muscle cell layer thickening and contraction are observed in the airways [40] (Fig.1).

1.1.2.1 Cellular inflammatory responses in allergic asthma

As previously mentioned, inflammatory cell infiltration of the lung is a major hallmark of asthma pathology. Cells that infiltrate the airways of asthmatic individuals include eosinophils, mast cells, and basophils [41]. These cells are hematopoietic in origin and are derived from granulocyte-monocyte progenitor cells

in the bone marrow [42, 43]. Following allergen exposure, cytokines and chemokines are produced locally in the lung, which recruit these cells and initiate allergic responses.

(1) Eosinophil

Eosinophils are multifunctional leukocytes involved in initiation and progression of diverse inflammatory responses including parasitic infection and allergic responses [44]. They are granulocytes that develop in the bone marrow and are released into the peripheral blood in their mature state, and they can be activated and recruited into local tissues by appropriate stimuli such as the cytokine IL-5 [45]. Human eosinophil granules contain proteins including eosinophil peroxidase, major basic protein (MBP), ribonucleases and eosinophil-derived neurotoxins. The granules also store numerous cytokines, enzymes and growth factors [45]. In allergic asthma, eosinophils undergo degranulation and release these proteins that cause epithelial cell damage, inflammation and airway remodeling [46]. It was also reported that activated eosinophils release reactive oxygen species [47] and important lipid inflammatory mediators including leukotrienes, prostaglandins, and thromboxanes [48, 49]. These mediators are shown to recruit and activate other inflammatory leukocytes like T cells and monocytes [50], resulting in vascular edema [51], smooth muscle contraction [52], and AHR [53].

Eosinophil infiltration to the airway is one of the first noted pathological changes in the asthmatics [54]. As the most predominant inflammatory cell involved in allergic asthma, eosinophils are observed in the peripheral blood or BAL among almost 70% of asthma patients [55]. Another study also found that eosinophilic

inflammation is correlated with asthma severity and a decline in pulmonary function [56]. Eosinophilic inflammation in clinical practice is also a reliable indicator of those patients who will benefit from treatment with inhaled corticosteroids. It was observed that asthmatics with eosinophilic inflammation respond better to steroids compared to those with non-eosinophilic inflammation [57]. However, the causal relationship between eosinophils and allergic asthma is conflicting. Studies using several approaches to suppress eosinophils including antibody against CCR3 (a chemokine receptor whose ligands are specific for eosinophils) for ablation of eosinophils in mice [58], IL-5 deficient mice [59] and eosinophil deficient mice [60, 61] have shown remarkable reductions in allergic inflammation and airway remodeling, but not complete inhibition. Overall, as one of the most important leukocytes in allergic asthma pathogenesis, eosinophils contribute to the disease progression, however, blockade of eosinophils may not be sufficient to improve disease outcome.

(2) Mast cell and basophil

Mast cells and basophils are important leukocytes in allergic inflammation. Mast cells are tissue-based inflammatory cells of hematopoietic origin [42], and basophils are basophilic granulocytes circulating in the peripheral blood [62]. While these cells differ in many aspects, they also share several similarities, including expression of the high-affinity IgE receptor FcεRI on their surface, and the release of inflammatory mediators such as leukotriene and histamine in response to various stimuli [63].

Both mast cells and basophils are reported to contribute to allergic asthma [64, 65]. In asthmatics, they are activated by IgE antibody crosslinking of their high affinity IgE receptor upon allergen re-exposure, leading to degranulation of these cells and release of inflammatory mediators such as tryptase, chymase, carboxypeptidase A, histamine, prostaglandins, and leukotrienes [66-68]. As previously discussed, these mediators result in vascular edema, smooth muscle contraction, and AHR. Other than releasing these mediators, both mast cells and basophils are also known to contribute to the development of Th2 immune responses. It was reported that mast cell derived tumor necrosis factors (TNF) may enhance cytokine production by Th2 cells [69], and basophils were noted in multiple studies for their capability to secrete large amounts of IL-4 and IL-13 after FcεR1 crosslinking [70-73].

Although both mast cells and basophils are present during allergic inflammation, studies using *in vivo* mouse models devoid of mast cells indicate that these cells are not sufficient to drive asthma pathogenesis [74-77].

(2) Neutrophil

Neutrophils are known as the first responders to inflammatory stimuli such as bacterial infections [78]. However, their role in asthma is not clear. Excessive neutrophils in the airway have been reported in certain asthmatics as early as 1986 [79]. This type of asthma was referred to as non-eosinophilic asthma, a subtype of the disease based on neutrophilic airway inflammation, possibly triggered by exposure to bacterial endotoxin, air pollutants, and ozone, as well as viral infections [80]. Several other studies have reported that elevated BAL neutrophils were

positively correlated with asthma severity [81, 82]. This non-eosinophilic asthma is often associated with a Th17 immune response rather than classical Th2 immunity [83, 84]. These patients exhibit significant resistance to corticosteroid treatment [84, 85].

The mechanisms by which neutrophils are recruited to the airways during allergic inflammation and the mechanisms by which they may induce the symptoms of asthma are not clear. Earlier studies suggest that neutrophilic inflammation is likely mediated by IL-8 [80], IL-1β, TNF-α, and leukotriene B_4 [86]. Recent evidence shows that IL-17A is an important regulator of neutrophil recruitment, which might provide a mechanism for the recruitment of neutrophils in severe asthmatics [87].

Neutrophils are known to release cytotoxic mediators and proteolytic enzymes that contribute to inflammation and tissue remodeling [88]. Neutrophils may also contribute to the processing of interleukin 33 (IL-33), a major epithelial cytokine mediating the pathogenesis of allergic asthma [89]. In summary, neutrophils are likely to play an important role in certain aspects of asthma, but their exact role in asthma is yet to be determined.

1.1.2.2 Lung function alterations in allergic asthma

(1) Airway hyperresponsiveness (AHR) and lung function

The most common clinical manifestations of allergic asthma are increased airway reactivity to bronchoconstrictor stimuli and decreased lung function. AHR is defined as the excessive response of the airway to a variety of stimuli such as methacholine, histamine, cold air, or exercise [90]. The mechanisms for induction of

AHR by different stimuli may differ, but also share common pathways. For example, methacholine is known to bind and activate cholinergic receptors expressed by airway smooth muscle cells, resulting in airway smooth muscle contraction [91]. Other stimuli such as cold air and exercise have been observed to cause AHR [92].

Together with AHR, a decline in lung function (FEV1/FVC) due to airway obstruction has also been observed in allergic airway response. The most common approach of measuring lung function is using spirometry to determine the forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) [93]. In an earlier study examining lung physiology of asthmatic patients, the mechanical function of the lung assessed with this approach showed a 50% to 60% reduction of predicted normal values due to persistent airflow obstruction in asthmatic peripheral airways [94]. With this observation, lung function measurement has been used as a clinical indicator for asthma, and a FEV_1/FVC ratio less than 70% is considered an indicator of airway obstruction. Generally, both methacholine challenge test (AHR) and FEV_1/FVC measurements (airway obstruction) are utilized in the clinical diagnosis of asthma.

(2) Airway remodeling

Besides the functional changes of the airways observed in asthmatics, structural alterations in the lung are also common in asthma patients [95]. The pathological changes reflecting airway remodeling, especially the altered bronchial epithelial barrier, were first described nearly 100 years ago [54]. In general, airway remodeling has been extensively studied and defined by series of structural cell and tissue alterations in asthmatics compared to healthy individuals such as airway wall

thickening, increased smooth muscle mass, mucus metaplasia, and subepithelial fibrosis [95].

Airway wall thickening is one of the most obvious pathological observations reported in almost all patients with asthma [96, 97]. However, it differs in fatal and non-fatal asthmatics: the airway thickening is most marked in the small cartilaginous and large membranous airways in fatal asthma, while it occurs more frequently in small membranous airways and mid-sized airways in non-fatal asthma [98]. The level of thickness was also found to be associated with the severity of the disease [96].

Airway thickening is due to several features including an increase in airway smooth muscle, edema, inflammatory cell infiltration, glandular hypertrophy and connective tissue deposition [95]. Airway thickening enhances airway lumen narrowing [97], and more importantly, it may uncouple the airway from the distending and tethering forces of surrounding lung parenchyma, leading to greater smooth muscle contraction and AHR [99]. As a main contributor to AHR, airway smooth muscle has been reported to undergo hypertrophy in asthmatics [100]. There are also studies speculating that the sensitivity of smooth muscle cells to contractile mediators might be higher in asthmatic patients [101].

Excessive mucus production by goblet cells is another important manifestation of asthma attacks [102]. Mucus is secreted by goblet cells in the airway epithelium and plays an important role in host defense [103]. However, in the remodeled airways of asthmatics, the mucus occupies a large percentage of the airway lumen and contributes to the narrowing and airflow resistance in airways

[102]. Studies have also shown that the area of mucous glands is increased in asthmatics [104]. Increases in mucus viscosity can also increase the surface tension at the air-liquid interface of the airways, thereby adding to the forces that tend to constrict the airways [105].

Increased deposition of extracellular matrix proteins such as collagen I, III, V, and fibronectin, referred to as subepithelial fibrosis, has been shown in asthmatics [106]. Subepithelial fibrosis is associated with a decline in FEV_1 [107], increased sensitivity to methacholine challenge [108, 109], and correlates with disease severity. However, subepithelial fibrosis was reported to be protective in some patients with severe asthma [110] probably due to its mechanical protection against smooth muscle contraction by providing adverse parenchymal pressure [105]. This indicates that the role of subepithelial fibrosis in asthma is more complicated than originally hypothesized.

In conclusion, although the exact mechanisms underlying asthma are not well understood, airway remodeling likely contributes to the airway narrowing observed during asthma attacks and exacerbations. Such constriction can be induced by neuro-transmitters such as acetylcholine from parasympathetic nerves [111], inflammatory mediators like histamine and leukotrienes from mast cells [65] and basophils [112], or by T cell cytokines such as IL-13 [113].

1.2 Immune responses in allergic asthma

1.2.1 Role of CD4+ T cells in asthma

Allergic asthma is thought to arise as a result of inappropriate immune responses to inhaled aeroallergens in susceptible hosts, and aberrant T cell responses are observed in asthmatic patients [3]. In particular, CD4+ T cells which orchestrate specific immune responses are thought to play an important role in asthma [114].

T cells originate from the bone marrow and are derived from T-lymphoid progenitor cells in the thymus through highly regulated subsequent differentiation processes [115]. These differentiation processes yield T cells expressing CD4 and CD8 on their surfaces [115, 116]. CD8+ T cells are primarily involved in the recognition and clearance of pathogens [117], but their roles in allergic asthma are rather controversial as studies have reported both protective [118] and pathogenic [119] outcomes after CD8+ T cell depletion.

Despite the unclear roles of CD8+ T cells in allergic asthma, CD4+ T cells are known to play a major role in the pathogenesis of allergic asthma. They were first shown to be elevated in acute severe asthmatics. Significantly increased expression of activated CD4+ T cell surface proteins (interleukin-2 receptor, class II histocompatibility antigen, and "very late activation" antigen) was observed in the serum of asthmatics compared to healthy individuals, which is decreased in patients with improved symptoms after treatment [120, 121]. The numbers of CD4+ T cells in BAL fluids and bronchial biopsies are increased in asthmatics as compared to healthy individuals [3, 122-124]. Also, CD4+ T cells are actively recruited to the airways following allergen challenge as a simultaneous drop in their number is observed in peripheral blood [125]. In addition to the evidence in human

asthmatics, depletion of CD4+ T cells with anti-CD4 antibody prior to antigen sensitization and challenge resulted in diminished allergic airway responses in mice [126]. Taken together these data supports a role for CD4+ T cells in disease pathogenesis.

CD4+ T cells are characterized by their distinct abilities to produce cytokines and function in the control of specific pathogens or immune responses. It is concluded that: Th1 cells are programmed through the transcription factor, T-bet. They produce interferon (IFN) γ in defense against intracellular pathogens. Th2 cells are programmed through GATA-3 and produce IL-4, IL-5, and IL-13 to defend against extracellular parasites. The newly identified Th9 cells, mediated by the transcription factor PU.1, produce IL-9 and play important roles in the regulation of cancer. Th17 cells are regulated by RORγt and produce IL-17 and IL-22 to mediate autoimmunity and immune responses to fungi. Lastly, regulatory T cells (Tregs) are induced by the transcription factor FOXP3, and they produce cytokine IL-10 and TGF-β to regulate priming and function of other T cells (see recent review [127]). Overall, these CD4+ T cells are highly regulated and play essential roles in all aspects in immunity. These specific CD4+ T cell subtypes play differential roles in allergic asthma [125, 128, 129], which will be discussed below in detail.

1.2.1.1 Th2

Th2 cells are known to play a central role in allergic asthma. It was initially observed that asthmatics displayed the pattern of cytokines that mirrors a Th2 phenotype in lung tissue and BAL [114, 125]. There were increased numbers of IL-4

and IL-5 producing CD4+ T cells in the BAL and biopsies from mild to moderate asthma patients compared to biopsies from non-asthmatic individuals [125]. Elevated levels of Th2 cytokines IL-4, IL-5 and IL-13 have also been confirmed in peripheral blood and BAL [114]. Later studies found that GATA-3 expression was higher in the airways of allergic asthmatics compared to healthy individuals [130]. Also, genetic analyses in humans have shown associations of polymorphisms in IL-13, IL-4 and Stat6 with asthma [131-133].

In addition to the evidence in human disease, studies in mouse models of disease have also provided definite support for the role of Th2 cells in asthma. Studies with genetic manipulation of IL-4 showed that depletion of IL-4 resulted in attenuation of allergic airway inflammation caused by OVA [134], while overexpression of IL-4 led to enhanced allergic responses [135]. Although it appears that IL-4 is playing a major role in allergic inflammation, antibody neutralization of IL-4 cannot effectively diminish allergic airway responses if given only during the allergen challenge. Furthermore, blockade of IL-4 has little effect on eosinophilia even if given during the systemic immunization period [136]. IL-5 is known to play a major role in the induction of eosinophilia. However, IL-5 antibody blockade in mice showed significant reduction of eosinophils, but no reduction in AHR [137].

Although both IL-4 and IL-5 play crucial roles in allergic inflammation, it was interesting to observe that blockade of the IL-4 receptor (IL-4R) at the time of allergen challenge was effective in inhibition of allergic phenotype [138], which seemed inconsistent with the earlier IL-4 antibody neutralization study. This led to the proposed mechanism that the IL-4R might have another ligand that played an

important role in generating the allergic airway responses. And indeed, IL-13, another Th2 cytokine, shared a receptor with IL-4 (i.e. composed of the IL-4Rα and the IL-13Rα1) [139]. Later studies suggested that IL-13 was the central player in allergic asthma [113, 140] by mediating the effector phase of allergic airway responses. *In vivo* blockade of IL-13 reversed allergen-induced allergic airway responses in mice, and administration of recombinant IL-13 or the overexpression of IL-13 in the lung was sufficient to induce allergic airway responses such as AHR, eosinophilia, and excessive mucus production [113, 140, 141]. In humans, candidate gene studies also identified SNPs in IL-13 as strongly associated with the risk of development of allergic asthma [133, 142]. Clinical trials using monoclonal antibodies to IL-13 have shown improved lung function in allergic asthma patients [143], and targeting the IL-4 receptor α complex also led to diminished allergic asthma symptoms [144, 145].

IL-13 is believed to act on resident airway cells to induce the pathological features of allergic asthma. It regulates mucus production via direct induction of mucin gene expression and mucus secretion *in vitro* in epithelial cell lines [146] and is likely mediated by Stat6 signaling [147]. Further studies confirm that IL-13 plays a pivotal role in mucus cell metaplasia, mucin gene induction, mucus packaging and release, and viscosity changes of the mucus produced *in vivo* [148]. IL-13 also contributes to sub-epithelial fibrosis [141], probably through induction of arginase 1 and TGF-β production by alternatively activated macrophages (M2 macrophages) and myeloid cells [149-151], as well as Stat6-mediated myofibroblasts proliferation [152]. More importantly, IL-13 can induce AHR via direct effects on resident airway

cells. Although the exact mechanism is not clear, it is generally believed that IL-13 directly enhances force generation and contractile function of airway smooth muscle cells in response to stimulation [153], and indirectly promotes airway smooth muscle cell proliferation and thus the muscle mass [154]. Despite the effects on airway smooth muscle cells, IL-13 also acts on bronchial epithelial cells and induces a series of signaling pathways that lead to the narrowing of the airways [148]. In conclusion, experimental data and clinical trials to date suggest that Th2 immune responses are a major driver in allergic asthma inflammation.

1.2.1.2 Th17

Th17 cells are a distinct lineage of CD4+ effector T cells that express IL-17A and IL-17F and play pivotal roles in tissue inflammation [155, 156]. They differentiate in response to IL-6, TGF-β and IL-23 through activation of Stat3, RORγt and RORα [157]. Despite their significant roles in autoimmune diseases, they are also associated with severe, neutrophilic, corticosteroid resistant asthma [158-160]. Asthmatics with severe, persistent disease exhibit neutrophilic infiltration rather than eosinophilic inflammation [161-163]. Further murine studies have shown that IL-17A drives neutrophil recruitment and AHR [164, 165]. Likewise, a study of allergic responses in RORγt transgenic mice confirmed a role for Th17 cells in the pathogenesis of severe asthma [166]. Specifically, RORγt transgenic mice had greater AHR and neutrophilic inflammation compared to wild type (WT) controls in response to OVA [166]. More importantly, such responses could only be ameliorated by neutralization of IL-17A, but not steroid treatment.

Th17 cells also contribute to airway remodeling. Th17 cells release IL-17A, IL-17F and IL-22 that promote proliferation of human airway smooth muscle cells [167]. Moreover, adoptive transfer of Th17 cells into allergen-exposed mice resulted in increased mucus production, airway smooth muscle mass, and peribronchial collagen deposition, which were abrogated by IL-17 neutralization [168].

Although it is possible that IL-17A alone drives allergic disease, it has also been reported that IL-17A is only capable of driving allergic asthma synergistically with IL-13 in allergen-exposed mice [169]. Another study also showed that IL-23 and Th17 cells could enhance Th2 cells-mediated allergic airway responses such as eosinophilia and AHR [170]. Together, these studies suggest that Th17 cells, rather than driving asthma pathogenesis, exacerbate established Th2 inflammation and asthma pathology. However, several studies also indicate protective roles of Th17 cells in allergic asthma pathology [171, 172]. Further studies are needed to elucidate the exact role of Th17 cell derived cytokines in allergic asthma.

1.2.1.3 Th1 and Treg

Besides Th2 cells and Th17 cells, other subtypes of CD4+ T cells also play a role in allergic asthma. Th1 cells, which are known to mediate immune response against intracellular pathogens [173], are generally thought to be protective in allergic asthma pathogenesis [174]. Th1 cells produce IFNγ, and their differentiation requires IL-12 [175]. It is believed that the transcription factor T-bet is the main regulator of Th1 cell differentiation by mediating expression of Th1 cell hallmarks such as IFNγ and IL-12 receptor expression [176].

Th1 cells were thought to play protective roles in asthma pathogenesis as identified by murine model studies [177]. The protective role of Th1 in allergic asthma is thought to be conferred via inhibitory effects on Th2 cell differentiation [174]. Studies show that T-bet inhibits the action of GATA3, the Th2 cell transcription factor [178]. Adoptive transfer of allergen specific Th1 cells also result in suppression of allergen-induced allergic airway inflammation [179]. However, contradictory results are also reported that Th1 cells could even enhance Th2 dominant lung inflammation [180]. Several human studies indicated that IFNγ levels correlate with asthma severity [181]. Moreover, in a longitudinal birth cohort study, it was reported that IFNγ levels were positively correlated with both atopic and non-atopic AHR [182]. In a murine model of asthma, adoptive transfer of antigen-specific Th1 cells was shown to mediate development of steroid–resistant AHR upon subsequent allergen and endotoxin lipopolysaccharides (LPS) challenge [183]. Indeed, a clinical study in which IFNγ was instilled into the lungs of asthmatics had no beneficial effect [184], indicating that Th1 cells are unlikely a good therapeutic target for allergic asthma.

As another important subtype of CD4+ T cells, Tregs were discovered and noted in many immunological disorders for their potent regulatory effects on multiple immune responses [185]. There are several subsets of Tregs, but they are generally recognized by the expression of the transcription factor Foxp3 which plays an important role in the differentiation and function of these cells, and release of the potent regulatory cytokine IL-10 [186]. These cells are highly plastic just as

the other CD4+ T cells, and studies found that attenuation of Foxp3 expression in these cells results in their conversion to Th1 or Th2 cells [187].

It is commonly accepted that Tregs represent the major mechanism of peripheral tolerance through the secretion of the anti-inflammatory cytokine IL-10 [188]. Reduced or altered function of Treg cell populations has been observed in asthmatics, and an inverse association between IL-10 levels and the severity of allergic asthmatic has been reported [189]. Multiple studies reported that transfer of Tregs attenuates allergen-induced allergic airway responses [190-192]. On the other hand, depletion of Tregs leads to exacerbation of experimental asthma [193]. The protective actions of Tregs against allergic inflammation are mediated by both direct and indirect mechanisms (see review [194]). They produce the anti-inflammatory cytokines, IL-10 and TGF-β, which significantly inhibit the inflammatory programming mainly by dendritic cells (DC) and T cells. They also express inhibitory molecules such as CTLA4 that could significantly reduce the activity of antigen presenting cells [195]. Indirectly, they may form direct cell contact with DCs via lymphocyte function-associated antigen 1 (LFA1) and CTLA4, leading to the down-regulation of dendritic cell derived co-stimulatory molecules [196]. Several studies have shown that the ability of Tregs to suppress Th2 cells is impaired in asthma patients, highlighting the role of Tregs in allergic asthma [129, 197, 198].

1.2.2 Initiation and amplification of type-2 immune responses

Although Th2 cells play a pivotal role in allergic asthma, their activation and amplification are largely controlled by antigen-specific recognition and presentation

[36]. The direct regulators of Th2 cell development are APCs, which control the phenotype and magnitude of resultant adaptive responses by interaction of the expressed MHC II molecules with T cell receptors (TCR).

Several cell types are recognized as APCs, including “professional” APCs such as DC, macrophages and B cells. These cells constitutively express major histocompatibility complex class II (MHC II) molecules and function readily upon taking up antigens [199]. Several “non-professional” APCs have also been reported according to their expression of MHC II including mast cells [200], basophils [201], epithelial cells [202] and innate lymphoid cells (ILC) [203]. Although MHC II is considered necessary for antigen presentation, cells must be able to take up and process antigens, migrate to secondary lymphoid organs, and express co-stimulatory molecules in order to effectively activate naïve T cells, making the “professional” APCs main effective presenters of antigens [199].

Antigen presenting cells are normally present in the lung and sample antigens from both the airway epithelium and the environment directly by uptake and transport of macromolecule antigens to the lymph nodes [204]. Taking DCs as an example, several activation signals are required for them to act as antigen presenting cells such as the expression of maturation markers [205] and activation of surface pattern recognition receptors (PRRs) [206]. Specifically, in the event of inflammation, immature DCs in the periphery are quickly recruited to the site of inflammation by chemokines produced by local tissues. These immature DCs are then exposed to antigens that can be recognized by their PRRs. Antigen uptake and PRR activation then result in increased expression of MHC II [207] and co-

stimulatory molecules such as CD80/86 [208], CD40 [209], and Ox40L [210]. These activated DCs then migrate to the draining lymph nodes where they interact with naïve T cells [211].

At this time, the antigens are presented to CD4+ T cells by DCs with the expression of MHC II molecules. MHC II proteins are encoded by three polymorphic genes HLA-DR, HLA-DQ, and HLA-DP in humans [212]. They bind the antigen peptide that is generated in the endosome of antigen presenting cells and transported to the plasma membrane [213, 214]. This process is referred to as the MHC II loading of antigens. DCs then interact via these loaded MHC II molecules with antigen-specific TCRs expressed by naïve T cells. Another signal required at this stage is the expression of co-stimulatory signals [215]. It has been reported that certain co-stimulatory molecules such as OX40/OX40L and CD40/CD40L are implicated in the induction of Th2 responses [216, 217].

After the activation and migration of DCs to the lymph node, the third signal required for effective T cell polarization is a Th2 cytokine promoting rich environment. Interestingly, DCs only produce required cytokines for Th1 (IFNγ/IL-12) and Th17 (IL-6/IL-23) differentiation [218], but not those required for Th2 differentiation. Previously, it has been proposed that the induction of Th2 immunity was a result of the absence of Th1 and Th17 polarizing signals [219]. This theory was proven incorrect as later studies suggested that basophils would provide the required co-stimulation as well as IL-4, for the induction of Th2 immunity [201]. However, a recent study suggested that it was in fact a subtype of inflammatory DCs accounting for Th2 induction rather than basophils, as basophils could not

perpetuate Th2 allergic immune responses in a model of HDM-induced experimental asthma [220]. Our knowledge of the factors required for the initiation of Th2 immunity has rapidly grown over the last few years, as type-2 innate lymphoid cells (ILC2s) have been found to be recruited early to the lung in response to papain exposure and provide IL-13 to boost DC function (mainly migration) and promote Th2 cell responses [221]. Moreover, eosinophils recruited into tissues and lymph nodes can also be a source of IL-4 and can promote DC-driven immunity [222]. As a result, basophils, eosinophils, and ILC2s all contribute to the initiation of Th2 immunity. This adds to the complexity of Th2 cell induction, and initiating events resulting in specific recruitment and activation of these cells following antigen exposure remain to be identified.

1.2.3 Epithelial cell regulation of the allergic response

Recent evidence suggests that airway epithelial cells are important players in allergic asthma. They lie at the interface between the host and the environment, and represent the first barrier of defense against external environmental stimuli [223]. Beyond their role as a passive physiological barrier, the airway epithelium is now perceived as an active interface between the immune system and the outside environment that shapes and regulates immune responses to external stimuli, e.g., type-2 immune responses against allergens and parasitic infections.

First, it was proposed that environmental factors causing disruption of the airway epithelium barrier might contribute to allergic inflammation [224]. As mentioned earlier, asthma patients have fragile and altered airway epithelium. This

is mainly because of the loss of epithelial barrier integrity through suppressed expression of E-cadherin, a major component of adherent junctions and airway epithelium [223]. Studies suggest that the loss of the epithelial barrier is a result of epithelial-to-mesenchymal transition (EMT) [225], a phenomenon in which mature epithelial cells change into a mesenchymal phenotype following morphogenic pressure from injured tissue [226]. This process not only contributes to airway epithelium associated remodeling, but may also promotes DC activation and allergic inflammation by allowing efficient DC encounter of antigens, and following antigen presentation [227].

Another more important mechanism by which the airway epithelium regulates the initiation of immune responses is via production of chemokines and cytokines that recruit and activate APCs for induction of Th2 immunity. The main epithelial-derived factors playing important roles in the downstream allergic response include: granulocyte macrophage-colony stimulating factor (GM-CSF), CCL20, IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) [227]. GM-CSF is released from epithelial cells in response to environmental stimuli such as diesel exhaust [228], particulate matter [229] and HDM [230]. It was found to effectively induce DC development *in vitro* [231] and was associated with Th2 polarized airway inflammation *in vivo* [230, 232]. CCL20 was also induced by HDM [233] and was shown to guide the accumulation of immature DCs expressing it receptor CCR6 [234]. IL-33, IL-25, and TSLP are mainly involved in the recruitment and differential activation of ILC2s [235], the newly identified important players in the initiation of the allergic response [236]. Among these cytokines, IL-33 can also act on DCs to

promote Th2 responses, as IL-33-treated DCs have been shown to induce robust IL-5 and IL-13 production by co-cultured T cells [237].

1.2.3.1 The epithelial-cell derived factor, IL-33

IL-33 is a cytokine belonging to the IL-1 family [238]. It has a β-trefoil fold in its carboxy-terminus similar to the other IL-1 members, allowing its binding to the orphan IL-1 receptor (IL-1R) family member ST2/IL1RL1 (ST2) [238]. It has been shown to activate Myd88-dependent signaling pathways in target cells expressing the ST2/IL-1RAcP receptor complex [238, 239] and induce subsequent production of pro-inflammatory mediators. IL-33 is constitutively expressed and located mainly in the nuclei of tissue structural cells including epithelial cells, endothelial cells and fibroblasts [240]. It was originally thought to function as an "alarmin" as it is released during cell necrosis, while cleaved and inactivated by caspases during apoptosis [240-242]. It was also found to be cleaved by calpain as well as neutrophil elastase and cathepsin G to generate its mature forms from the full-length protein with enhanced biological activity [243]. The regulation of IL-33 remains poorly identified, but recent evidence suggests that besides its alarmin properties, it is also released upon environmental exposures to allergens, virus and mechanical stress [244-247].

(1) Regulation of IL-33 release

Although IL-33 has been recognized as a major player in type-2-mediated allergic inflammation, the exact pathways regulating its production, transport within the cells, and extracellular release are not well understood. Although it was thought

to function as an alarmin following cellular damage/injury [248], extracellular danger signals or environmental stimuli can also induce live cells to release pre-formed IL-33 within minutes by airway epithelial cells and fibroblasts from both the nuclei and other vesicular compartments where this cytokine is located [244-247]. This was confirmed *in vivo*, as the increase of IL-33 protein in extracellular fluids is associated with reduced staining for IL-33 protein in the nucleus [249].

Initially, researchers attempted to identify a signaling peptide within the IL-33 protein. Signaling peptides are specific peptide sequences within a protein molecule, allowing the protein to be recognized by the shuttling machinery within conventional endoplasmic reticulum (ER) and Golgi cisternae-mediated secretory pathways [250]. However, IL-33 does not have a traditional signal sequence [251]. Recently, researchers found that IL-33 was located both in the nucleus and in membrane-bound cytoplasmic vesicles in fibroblasts [247]. They determined through fluorescent pulse-chase fate-tracking experiments that there was dynamic nuclear-cytoplasmic flux of IL-33, which was reduced after cytoskeleton disruption with latrunculin B [247]. These studies indicated that IL-33 translocation between intracellular organelles is mediated by cytoskeleton rearrangement. Another two groups found that endogenously produced ATP and its autocrine activation of purinoceptor-dependent signaling and calcium mobilization was required to translocate IL-33 to extracellular milieu [244, 245]. They further showed that the elevated intracellular calcium resulted in dual oxidase-1-mediated activation of calpainin, a protease thought to process IL-33 [243, 245]. Taken together, these studies support the concept that inter-organelle translocation of IL-33 in airway

epithelial cells is dependent upon cytoskeletal rearrangement and calcium signaling. However, to date, no direct evidence for the regulation of IL-33 live cell release or translocation has been provided.

(1) Regulation of IL-33 activity

Another level of IL-33 regulation is its processing by caspases and proteases. IL-33 was found to contain a cleavage site for caspase-3, which could cleave IL-33 and generate two biologically inactive products [252]. This was probably a protective mechanism to avoid unnecessary activation of its downstream immune responses. However, the role of caspases in inactivating IL-33 remains controversial, as another study found that the release and activity of IL-33 from macrophages and mast cells is independent of caspases [252].

However, as the knowledge of IL-33 processing has evolved, evidence has accumulated to suggest that the mature form of IL-33 is formed through enzymatic cleavage [89, 243, 245]. Specifically, the cleaved forms of IL-33 (IL-$33_{95–270}$, IL-$33_{99–270}$ and IL-$33_{109–270}$) generated by neutrophil elastase and cathepsin G *in vitro* and *in vivo* have 10 fold more biological activity compared to the full-length protein in their capability to induce granulocytes and monocyte production of pro-inflammatory cytokines [89]. However, the roles of proteases derived from granulocytes in IL-33 activity regulation remain controversial, as the mast cell chymase is able to degrade IL-33 [253], and neutrophil proteinase 3 (PR3) cleavage of IL-33 actually results in the generation of two inactive truncated forms of IL-33 *in vitro* [254]. Taken together, these data suggests a complex balance of maturation and inactivation of IL-33 in local tissues that affects its biological function.

1.2.3.2 IL-33 and type-2 immune response

IL-33 plays a pivotal role in allergic inflammation. Current studies have suggested a role for IL-33 the regulation of both innate and adaptive arms of type-2 immune responses.

(1) DCs

Accumulating evidence suggests that DCs express the IL-33 receptor ST2 [237, 255, 256]. As professional antigen presenting cells, DCs are critical in controlling adaptive T helper cell responses. Initial studies demonstrated that IL-33 treated DCs polarize naïve CD4+ T cells into Th2 cells which produce IL-5 and IL-13 but not IL-4, while IL-33 treatment of CD4+ T cells alone is not sufficient to induce these Th2 cytokines [237, 255]. In the meantime, ST2 knockout (ST2 -/-) mice exhibit attenuated allergic inflammation in the lung after allergen exposure, probably through diminished activation and migration of DCs in these animals [255]. Interestingly, DCs can be driven to express IL-33 upon stimulation by allergen-IgG immune complexes [257] or TLR ligands [258]. In one study using HDM as the stimulus, delivery of IL-33 restores the attenuated allergic inflammation in FcγR-/- mice, and the same group reveals that DC *Il33* expression is dependent on FcγR, TLR4, and PI3K signaling [259].

(2) Macrophages

As previously discussed, macrophages also play a role in allergic asthma. M2 macrophages that are generated in the presence of IL-4 and IL-13 are known to contribute to tissue remodeling [260]. IL-33 administration was shown to promote

macrophage associated TLR signaling [261], as well as polarization into the M2 subset and airway inflammation [262]. Thus, IL-33 enhances the function of M2 macrophages in Th2 promoting environments. However, despite regulation of macrophage function, recent evidence also shows that macrophages may be an important source of IL-33 [263, 264]. The exact mechanism that drives macrophage expression of *Il33* is not clear, but it is probably an important driver of M2 macrophage-mediated pulmonary fibrosis in acute lung injury induced by bleomycin [263], suggesting that it may also contribute to the airway remodeling in allergic airway inflammation where fibrosis is also implicated [95].

(3) Mast cells

Mast cells are critical effector cells in allergic asthma. Acute IL-33 exposure potently activates ST2 expressing mast cells both dependent upon and independently of IgE [265, 266]. In the IgE independent activation of mast cells, IL-33 binds ST2 and leads to activation of NF-κB and p38 MAPK signaling [267, 268], resulting in expression of Th2 cytokines IL-4, IL-5 and IL-13 [269, 270]. As a result, IL-33 enhances the capacity of mast cells to intensify type-2 immune responses within the tissue. In the IgE dependent activation of mast cells, IL-33 synergizes with IgE to cause enhanced mast cell degranulation and cytokine synthesis [266, 268]. IL-33 also increases IgE synthesis from plasma cells [269], leading to further intensified mast cell activation. Additionally, IL-33 promotes mast cell proliferation, survival or maintenance upon allergic inflammation. For example, an early study showed that the size of the mast cell population in IL-33 -/- mice is smaller than that

of wild type controls following helminth parasite infection [271]. These findings suggest another mechanism through which IL-33 might magnify mast cell activity.

The crosstalk between IL-33 and mast cells is rather bi-directional, as mast cells are also critical regulators of IL-33. Mast cells express IL-33, and their activation causes increased *Il33* mRNA expression [272] and release that is detectable in the *in vitro* culture supernatants [273, 274]. Such a phenomenon demonstrates a potential autocrine mechanism through which inflammatory cell-release of IL-33 may in fact cause further activation of these cells via activation of ST2, resulting in an even greater magnitude of the inflammatory response. Besides being a source of IL-33, mast cells may also control the duration and potency of IL-33 protein activity. For example, mast cell degranulation leads to the release of serine proteases, trypase, and chymase, which are shown to cleave and process full-length IL-33 protein, resulting in sub-forms with greater biological activities that contribute to allergic airway inflammation [275, 276].

However, IL-33 doesn't always lead to greater activity of mast cells. Long-term IL-33 exposure impairs downstream signaling of FcεRI activation, which down-regulates mast cell degranulation [277]. Whether the Th2 cytokine release can be suppressed after long-term IL-33 exposure remains unknown.

(4) Eosinophils, basophils, and neutrophils

Eosinophils are the "classical" magnifier of type-2 immune responses. A recent study shows that eosinophils also express ST2, and are rapidly activated by IL-33 to degranulate and release Th2 cytokine [278]. This is likely caused by NF-κB-dependent gene transcription, which is downstream of ST2 signaling, and the

activation of eosinophils by IL-33 is likely more effective than that by IL-4 [279]. IL-33 also facilitates eosinophil migration mediated by ICAM-1 [280]. Together, the data suggest that IL-33 enhances eosinophil recruitment and activation in allergic inflammation. Similar to eosinophils, IL-33 also binds ST2 and enhances IgE initiated basophil degranulation and release of pro-inflammatory mediators [281]. By activation of PI3K/Akt pathway, IL-33 could promote basophil expansion and survival [282, 283].

While neutrophils are implicated only in certain subtypes of asthmatics, they also interact with IL-33 and are potentially linked to allergic inflammation. Neutrophils could generate proteases, elastase and cathepsin G, which are known to process full-length IL-33 into more potent forms [89]. IL-33 may also enhance neutrophil recruitment by driving release of neutrophil chemotactic factors from mast cells [284] and macrophages [285]. This indicates that neutrophils recruited in asthmatics may enhance IL-33 activity and contribute to disease pathogenesis.

(5) ILC2s

ILC2s are key activators of type-2 immune responses that are recruited and activated by IL-33. They are defined based on: (1) their lack of lineage markers in combination with expression of cell surface markers including ST2 [286, 287], (2) the production of type-2 cytokines in response to epithelial-derived IL-25, IL-33, and TSLP [288-293], and (3) their dependency on the transcription factors RORα and GATA-3 for their proper development and function [294-297]. More specifically, they do not express the cell-surface markers of most immune cells such as T cells (CD3,

CD4), NK cells (CD49b), B cells (B220), DCs (CD11b, CD11c) and macrophages (CD11b, F4/80) [298].

ILC2s are hematopoietic in origin and are developed under the regulation of Interleukin-7 (IL-7), transcription factors inhibitor of DNA binding 2 (ID2), and nuclear factor IL-3 (NFIL3) as well as transcription factors GATA-3 and RORα [299, 300]. ILC2s are known to produce the Th2 cell-associated cytokines IL-5 and IL-13, but not IL-4, and to mediate allergen-driven airway inflammation [301]. As previously mentioned, these cells express ST2, and IL-33 has been shown to be a potent inducer of their expansion and secretion of Th2 cytokines. The induction of Th2 cytokines is mediated through the activation of numerous signaling molecules such as nuclear factor-κB (NF-κB), extracellular signal-regulated kinase 1 (ERK1), ERK2, p38 and JUN N-terminal kinase-1 (JNK1) downstream of ST2 ligation [288, 302, 303].

As a newly identified cell type, little is understood about the clinical relevance of ILC2 cells in human asthma. However, they are increased in the nasal polyps of a subset of severe asthmatics that often have comorbidity with chronic rhinosinusitis (CRS) and do not respond well to steroids [304, 305]. The IL-33/ILC2 axis has been widely implicated in mouse models of allergic airway inflammation [221, 306-308]. More importantly, ILC2s serve as potent innate producers of Th2 cytokines in that they constitute the majority of cells expressing IL-5 and IL-13 in the lung after HDM exposure or IL-25 and IL-33 administration [301]. Further studies reveal that pulmonary resident ILC2s responding to IL-25, TSLP, and in particular, IL-33, contribute to allergic airway inflammation and AHR independently

of the adaptive immune response [306-308]. Depletion of ILC2s abolished both viral and allergen-induced AHR, while adoptive transfer of purified ILC2s reconstituted AHR [307, 308]. All these studies suggest that ILC2s play a role in the early, innate response to environmental stimuli, providing an early source of Th2 cytokines and driving allergic inflammation. Besides, ILC2s also interact with other immune and structural cells by acting as MHC II-dependent antigen presenting cells *in vitro* to stimulate T cell responses [309], by driving Th2 cytokine secretion by CD4+ T cells via direct contact [310], by promoting B cell IgE production [311], and by contributing to pathological airway remodeling via amphiregulin production [312].

(6) CD4+ T cells

As discussed earlier, CD4+ Th2 cells facilitate the pathophysiology of allergic asthma. Although naïve T cells and resting Th2 cells have very low-level expression of ST2, it can be induced by cytokines such as IL-2 in the presence of IL-33 [313]. These effects are likely direct as in the presence of antigen, IL-33 induces the production of IL-5 and IL-13, but not IL-4, by murine Th2 cells, which is sufficient to induce allergic airway inflammation [314]. Further studies suggest that IL-33 may also mediate the migration of Th2 cells: *ex vivo* studies show that IL-33 increases migration of Th2 cells isolated from human and mouse, but not in ST2 deficient Th2 cells [315].

In summary, IL-33 plays a crucial role in promoting type-2 immune responses and allergic asthma pathogenesis. It forms highly interconnected interactions with other drivers and effectors of the disease. As a result, dysregulation of IL-33-mediated pathways would result in local allergic

inflammation. The capacity of IL-33 to promote IL-13 production by immune cells, particularly ILC2s, is crucial for pathogen responses, but also detrimental in asthma pathogenesis.

1.2.3.3 Epithelial cell recognition of environmental allergens

As airway epithelial cells play important roles in the regulation of type-2 immune responses through the secretion of IL-33, the recognition of such environmental stimuli leading to IL-33 release most likely plays a critical role in the initiation of allergic inflammation. Airway epithelial cells respond to environmental stimuli through many PRRs, and the environmental stimuli recognized are termed pathogen-associated molecular patterns (PAMP) and danger-associated molecular patterns (DAMP) such as macromolecules with cabonhydrate moieties (lipopolysaccharide, peptidoglycan), proteins (flagellin), and nucleotide sequences (bacterial or viral DNA and RNA) [316].

There are many types of PRRs, which recognize a wide variety of stimuli [316]. PRRs are categorized according to their cellular locations, as membrane bound, secreted and/or intracellular. Membrane bound PRRs include Toll like receptors (TLRs), C-type lectin receptors (CLRs), protease-activated receptors (PARs), and a variety of scavenger receptors. Secreted PRRs include serum amyloid a (SAA), C-reactive protein (CRP), and mannose-binding lectin (MBL). Intracellular PRRs include nucleotide oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) [317]. PRR ligation results in the activation of downstream signaling pathways such as nuclear factor-kappa B

(NF-κB) and mitogen-activated protein kinase (MAPK) that result in the secretion of proinflammatory cytokines and chemokines [318].

Depending on the nature of the antigens, different PRRs initiate distinct immune responses. The identification of PRRs involved in the regulation of type-2 immune responses is lacking. As a major aeroallergen exhibiting great complexity of its contents, HDM is thought to activate multiple PRRs that together contribute to the activation of multiple innate immune pathways [319]. Indeed, several studies have attempted to identify the allergenic component(s) of HDM responsible for the induction of allergic asthma. For example, the HDM allergen, Der p 2, was found to interact with the TLR4 signaling complex by mimicking the structure of MD2, the lipid binding component of TLR4 signaling complex, to augment adaptive allergic responses [320]. However, this study did not explore the role of TLR4 in HDM induced IL-33 release from airway epithelial cells. In fact, there are few studies addressing this question except for one showing the involvement of the P2 purinergic receptor that recognizes extracellular ATP [244]. As a result, identifying the PRRs recognizing HDM allergens that lead to the release of the type-2 immune response regulators IL-33 remains a major priority.

Overall, disruption of the epithelial cell barrier function and activation of PRRs lead to epithelial cell activation and subsequent changes including secretion of soluble factors [227]. These soluble factors include cytokines such as IL-33, IL-25, TSLP, GM-CSF, CCL20, as well as DAMPs such as ATP, uric acid, and reactive oxygen species (ROS) [227]. Among these cytokines, IL-25, TSLP and IL-33 all play important roles in the initiation of allergic inflammation. Of these, IL-33 is one of the

major players. Understanding the HDM specific signaling that leads to IL-33 production by airway epithelial cells not only advances the current understanding of allergic asthma pathogenesis, but also provides future directions for the disease prevention and treatment.

1.3 Cellular glucose metabolism regulation of immune cell function

1.3.1 Cellular glucose metabolism

Cellular glucose metabolism has long been recognized as one of the most important physiological processes. Recent emerging evidence shows that cellular glucose metabolism regulates vital pathways for cell survival and activation [321, 322], extending the recognition of this old concept into new fields, such as its roles in the regulation of immune responses. The crosstalk between glucose metabolism and the immune system has provided great insights into the development, function and regulation of immune cells. Specifically, cell metabolism has been shown to regulate the synthesis and release of certain mediators and cytokines, conferring importance in pathological conditions and immune homeostasis [323-326].

The main purpose of cellular glucose metabolism is to produce energy and substrates for various cellular processes [327]. The major form of energy for cellular activities is ATP. To generate ATP, cells utilize primarily glucose, and sometimes fatty acids in the presence of oxygen [328]. Basically, glucose is taken up by cells via glucose transporters (GLUT). There are 12 members of the GLUT family proteins. While the primary glucose transporter expressed on mammalian cells is GLUT1, metabolic organs such as liver and pancreas may rely on other GLUT proteins for

functions such as glucose sensing, insulin responses and fructose uptake [329]. After cellular uptake, glucose is first metabolized to pyruvate via glycolysis catalyzed by a set of enzymes (Fig. 2). During oxygen deprivation, pyruvate can be converted to lactic acid (1 molecule of lactate and 1 molecule of proton) by lactate dehydrogenase (LDH) in the cytosol. This process is referred to as anaerobic glycolysis. During this process, a total of four molecules of ATP are formed from ADP in glycolysis. However, two ATP molecules are consumed during earlier steps of this pathway as shown in Fig. 2, resulting in the net production of 2 molecules of ATP per each molecule of glucose. When sufficient oxygen is available, pyruvate can also be fully oxidized to CO_2 and H_2O via the tricarboxylic acid (TCA) cycle in the mitochondria. This process involves reduction of O_2 to H_2O and activity of ATP synthase in the electron transport chain in the mitochondria, generating a total of 36 molecules of ATP [330]. These two processes are interrelated and integrated when pyruvate is converted into acetyl-CoA to enter the TCA cycle, and the balance of these two processes is regulated by the availability of oxygen and/or the energy demand for cellular events [330].

As discussed above, in the presence of sufficient O_2, ATP is mostly generated by oxidative phosphorylation (OXPHOS). Under hypoxic conditions cells mainly generate ATP via glycolysis. However, in some cases, cells may preferentially use glycolysis for ATP generation even in the presence of sufficient oxygen. This process is referred to as aerobic glycolysis or Warburg metabolism [331]. Moreover, it has been proposed that certain cellular events might use ATP from different sources based on their need. For example, glycolysis derived ATP can fuel the needs of

membrane-associated actions (those related to channels and transporters) in a timely manner compared to OXPHOS derived ATP [332]. This is probably explained by the fact that the immediate energy source for ATP synthesis in the mitochondria is provided by the proton-motive force across a membrane [328], which may not be as "convenient" as the substrate-level phosphorylation that is catalyzed by water-soluble enzymes in the cytosol independent of membranes and ion gradients [328]. As a result, glycolysis derived ATP is proposed to be generated physically in greater proximity to membrane compartments in a timely manner [332]. In addition, glycolysis and OXPHOS generate different intermediates, meeting different needs of cells through several key pathways such as the pentose phosphate pathway (PPP) for synthesis of pentose sugars, fatty acid synthesis, and lipid ligands production [325].

1.3.2 Glucose metabolism and immune cell activities

Crosstalk between cellular glucose metabolism and immune cell activities has been widely implicated. In the immune system, most cells cycle between a quiescent state and an activated state in response to perturbations of the host. During such transitions, large numbers of cellular events take place including gene expression and post-translational modifications, resulting in the production of cytokines, inflammatory mediators, tissue remodeling factors, as well as physical actions of migration and proliferation [325].

Cell metabolism has been shown to regulate activation of many immune cells include granulocytes [333-336], DCs [337, 338], macrophages [339, 340], T

cells [321, 341, 342] and B cells [343, 344]. For example, neutrophils and eosinophils are highly dependent on glucose for ATP production via aerobic glycolysis [334]. Upon activation by TLR agonists or cytokines, they exhibit increased consumption of glucose and metabolic activities [333, 335]. Similar observations were made with antigen presenting cells. Studies on cell metabolism in DCs found that while resting immature DCs rely mainly on OXPHOS as most tissue cells. TLR stimulation reprograms their metabolism and they become dependent on aerobic glycolysis and Warburg metabolism [337]. A recent study further showed that DCs undergo rapid induction of glycolysis activity that drives cytokine production and MHC molecule loading [338]. Similar to DCs, glycolytic flux is also very important for macrophage activation [339], while altered balance between OXPHOS and glycolysis is thought to regulate macrophage polarization between classic and alternative activations [345]. In T cells, the role of glucose metabolism is more extensively studied. Firstly, T cells engage Warburg metabolism upon activation for their extensive and rapid proliferation [321]. Studies have shown that molecules such as the mammalian target of rapamycin (mTOR), phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), Myc, and hypoxia-inducible factor (HIF) are all playing roles in the crosstalk between immune signals and metabolic cues for the activation, development, function, and maintenance of T cells [341]. In fact, mTOR signaling was shown to direct naïve T cell differentiation into Th2 cells, further confirming that metabolic alterations in T cells contribute to allergic asthma [346]. Likewise, B cells also share certain fundamental metabolic characteristics with the

other immune cells, such as their increased glucose uptake and induction of glycolysis after activation [343, 344].

While the most common hypothesis for the role of cell metabolism in immune cell activation is via satisfaction of the energy needs for immune cell activation, other studies have also explored potential mechanisms by which certain metabolites can be involved. For example, there are many G-protein coupled receptors (GPCR) that serve as receptors for metabolic intermediates and energy substrates [347]. Among these receptors, GPR81 recognizes lactate and has been shown to promote cancer cell survival [348], and GPR91 ligates succinate to promote chemotaxis and activation of DCs [349]. Moreover, the A2B and A2A adenosine receptors, when activated, are shown to strongly promote IL-4 driven alternative activation in macrophages [350]. Besides inducing signaling pathways in cells, metabolites may also stimulate immune cell responses. One example is extracellular ATP, which is released at the mucosal surface following tissue damage [351]. ATP was shown to act as a DAMP to induce IL-33 release from epithelial cells [244], and was found to be associated with allergic airway inflammation [352].

1.3.3 Regulation of glucose metabolism

As mentioned previously, glucose metabolism has been shown to play an important role in the activation and function of multiple cell types. Extensive studies have explored the potential pathways linking immune cell activation and altered glucose metabolism. It has been shown that activation of the AMP-activated protein kinase (AMPK), mTOR, as well as other molecules (c-Myc, hypoxia inducible factor-

1α (HIF-1α), etc.) may contribute to the glucose metabolism phenotypes in activated immune cells.

(1) AMP-activated protein kinase (AMPK)

AMPK is a key regulator of cellular metabolism. It promotes ATP conservation and production in times of ATP deficiency. Therefore, pathways producing ATP (glycolysis, fatty acid oxidation, etc.) are turned on, and pathways consuming ATP (gluconeogenesis, glycogen synthesis, etc.) are decreased [353]. AMPK also increases the activity of GLUT1, the major glucose transporter, to enhance glucose uptake [354]. AMPK is mainly activated in times of energetic deprivation, in which there is an increased AMP: ATP ratio in the cells [355]. During cell activation, the rapid increased demand of energy may result in activation of AMPK signaling. For example, AMPK is rapidly activated in T cells following TCR stimulation [354], leading to the previously described metabolic alterations in activated T cells.

(2) mTOR

mTOR signaling is shown to mediate cell metabolic alterations during cell activation and contribute to the activation process. mTOR exists as two complexes, mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2), and integrates extrinsic and intrinsic signals related to nutrient levels, energy status, and stress to induce changes in cellular metabolism, growth, and proliferation [356]. Extracellular stimuli activate PI3K, which recruits 3-phosphoinositide–dependent protein kinase-1 (PDPK1) and Akt. PDPK1, together with mTORC2, phosphorylates Akt, which in turn activates

mTORC1 [356]. Both Akt and mTOR promote aerobic glycolysis by regulating nutrient transport and phosphorylation of glycolytic enzymes [357]. mTORC1 could also increase protein translation [356] and lipid synthesis [358]. mTOR activation is implicated in effector T cell differentiation, growth, and function [359]. Specifically, Th1, Th2, and Th17 cells strongly engage glycolysis via mTOR signaling, whereas Treg cells depend more on the oxidation of lipids [360]. Other studies suggest that mTOR also play important roles in activation of DCs by TLR agonists [360].

(3) Others

Besides the above two master regulators of cell metabolism, many other pathways contribute to metabolic regulation of cell activation in a transcription-dependent fashion. For example, up-regulation of transcription factors c-Myc, estrogen-related receptor α (ERRα), and HIF-1α coordinately drives the expression of genes involved in metabolism alterations that fuel the rapid proliferation of effector T cells during clonal expansion [361]. In dendritic cell activation, mTOR could target HIF1α and mediate DC activation [362]. HIF1α induces the expression of many enzymes in the glycolysis pathway and promotes glycolysis in several systems [363]. However, in more immediate activation of DCs, the induction of glycolysis depends totally on post-translational modification. In this condition, TLR agonists activate TANK-binding kinase 1 (TBK1) and Akt, leading to a rapid increase of glycolysis and then DC activation [338].

Alterations in cell metabolism contribute to many aspects of cell activation. In most cases, activation of cell surface receptors (e.g., PRRs) and cytokine receptors

on immune cells leads to the activation of metabolic-related signaling pathways, which either induce or promote the activation and function of cells.

1.3.4 Glucose metabolism changes in Asthma

Based on the potential role that cellular metabolism plays in immune cell function, the role that altered glucose metabolism plays in allergic asthma pathogenesis has been recently explored.

Although studies have shown that metabolic alterations occur in cells relevant to asthma, it is not clear whether there is a correlation between such metabolic phenotypes and asthma in humans. The earlier reports about metabolism in asthmatics focused on lactic acidosis during asthma attacks, in which people found increased serum lactate levels in acute severe asthmatics despite normal oxygenation [364-369], which was thought to be attributed to increased cellular metabolism during asthma attacks as well as medication-related sympathetic effects. However, in the recent study conducted by Ostroukhova et al., they found that isolated CD4+ T cells from asthmatics produce more lactate than those from normal individuals, suggesting metabolic reprogramming in these cells. Interestingly, they observed that lactate treatment significantly increased T cell proliferation *in vitro*. When treated with a glycolysis inhibitor, cytokine production and proliferation of purified CD4+ T cells were significantly reduced, although there was no direct evidence for specific inhibition of Th2 cell activities. This study established a correlation between enhanced glycolysis and allergic inflammation in humans. In the same study, they also gave a glycolysis inhibitor to mice that were

sensitized to ragweed, and observed that while ragweed treatment significantly induced allergic airway responses in those mice, inhibition of glycolysis significantly reduced airway resistance, remodeling, and Th2 cytokine (IL-5, IL-13) levels in the lung [370].

Intriguingly, another study conducted at the same time attempted to understand the biological mechanisms involved in the early asthmatic responses following allergen exposure. Xu and colleagues treated rats with OVA, and harvested the lungs 14 days later to determine the protein expression profiles associated with allergic responses. They found that the rats exposed to OVA had significantly greater pulmonary resistance, and proteomic analyses of the lung proteins revealed an increased expression of proteins related to glycolysis (glucose transporter, pyruvate kinase, etc.), calcium signaling, and mitochondrial activities [371]. Another metabolomic study also found that OVA-sensitized mice had greater lactate levels in the BAL [372]. These studies provided further evidence that glycolytic activities are involved in asthma pathogenesis. Collectively, these data support the hypothesis that enhanced glycolysis is prevalent in allergic asthma, indicating that alterations in the balance of cell metabolism may be associated with the development of allergic asthma. In support of this concept, cell metabolism has recently been found to be involved in the activity and function of important cells for type-2 immune responses. For example, naïve T cell differentiation into Th2 cells is dependent on mTOC-mediated metabolic reprogramming [346]; glycolysis has been found to play an important role in immature dendritic cell activation and mature dendritic cell

survival [373]; B cells require metabolic reprogramming and rely on glycolysis for its proliferation and antibody production [374].

However, despite the accumulating evidence that changes in cellular metabolism may play a role in allergic inflammation, little is known about the cell metabolic phenotypes of residential structural cells in human asthma, and few studies have investigated whether metabolic reprogramming regulates type-2 immune responses. Considering that cell metabolism has been implicated as an important mechanism for immune cell activation and associated with allergic asthma, further analyses for its role in regulating initiation of the aberrant immune responses in allergic asthma are needed.

Airway epithelial cells play pivotal roles in regulating type-2 immune responses by the secretion of IL-33. As such, we propose to address the following questions in the current study: (1) does the common aeroallergen HDM induce metabolic alterations in airway epithelial cells? (2) Does cellular glucose metabolism contribute to IL-33 secretion by airway epithelial cells? (3) Does modulation of cellular glucose metabolism affect HDM-induced allergic inflammation *in vivo*? By answering these questions, we hope to advance our current understanding of the mechanisms driving the initiation of allergic asthma caused by the major allergen HDM, in order to provide insights into the development of novel asthma prevention and therapies.

1.4 Summary and hypothesis

Allergic asthma is a chronic disease of the lung, whose prevalence has increased dramatically over the last few decades. Although the etiology of asthma is not well understood, both genetic and environmental influences are known to contribute to its pathogenesis. Indeed asthma is thought to arise as a result of inappropriate immune responses to innocuous environmental allergens. Sensitization and challenge with allergens such as HDM results in aberrant type-2 immune responses that lead to the functional and pathological features of the disease including AHR, eosinophilic inflammation and elevated IgE synthesis. Recently studies have shown that the release of the cytokine IL-33 from bronchial epithelial cells is an important driver of type-2 immune responses and allergic asthma.

The mechanism(s) by which epithelial cells secrete IL-33 following exposure to environmental stimuli remain unknown. Recent studies suggest that asthma is associated with an imbalance between glycolysis and oxidative phosphorylation in the lungs, and cell metabolism related pathways are thought to play pivotal roles in early responses of immune cells to external stimuli. As IL-33 is rapidly released upon environmental exposure, it is likely that allergen-induced changes in epithelial cell metabolism also mediate IL-33 production. In this thesis, **we propose the novel hypothesis that environmental allergen exposure induces changes in glucose metabolism in airway epithelial cells, which modulates secretion of the Th2-promoting cytokine IL-33 and the development of allergic asthma.** To address this hypothesis, we propose the following specific aims:

1. **To determine whether HDM-induced metabolic changes in bronchial epithelial cells drive IL-33 secretion.**
2. **To delineate the mechanism(s) of HDM-induced glycolysis in bronchial epithelial cells.**
3. **To determine whether modulating glycolytic metabolism affects allergic asthma *in vivo*.**

1.5 Figures

Figure 1: An integrated view of allergic asthma initiation. This figure presents an overview of the cell types and cytokines involved in the initiation of allergic asthma. Briefly, allergic sensitization results from aeroallergen exposure and recognition of allergenic components by PRRs expressed on the epithelium. This leads to the release of soluble mediators such as IL-33, IL-25, TSLP, GM-CSF, and CCL20 that play different roles in the recruitment of inflammatory cells such as DCs and ILC2. ILCs release Th2 cytokines IL-5 and IL-13 that contribute to recruitment of additional inflammatory cells such as epsinophils, and contribute to DC-mediated Th2 cell polarization. DCs process and present the allergens to the naïve CD4+ T cells in the draining lymph nodes. In the presence of MHC II molecule, co-stimulatory molecules (co-stim) and Th2 cytokine, Th2 cells are differentiated. Th2 cells then interact with B cells, in combination with IL-13, leading to the production of IgE. IgE can then bind to the surface receptors on mast cells and basophils, leading to release of lipid mediators. In the meantime, IL-13 signals on macrophages, airway smooth muscle cells, and the airway epithelium to result in allergic asthmatic phenotype, marked by airway narrowing, airway hyperresponsiveness, mucus production, and elevated levels of the Th2 cytokines IL-4, IL-5, and IL-13, as well as increased levels of serum IgE.

Figure 1: An integrated view of allergic asthma initiation.

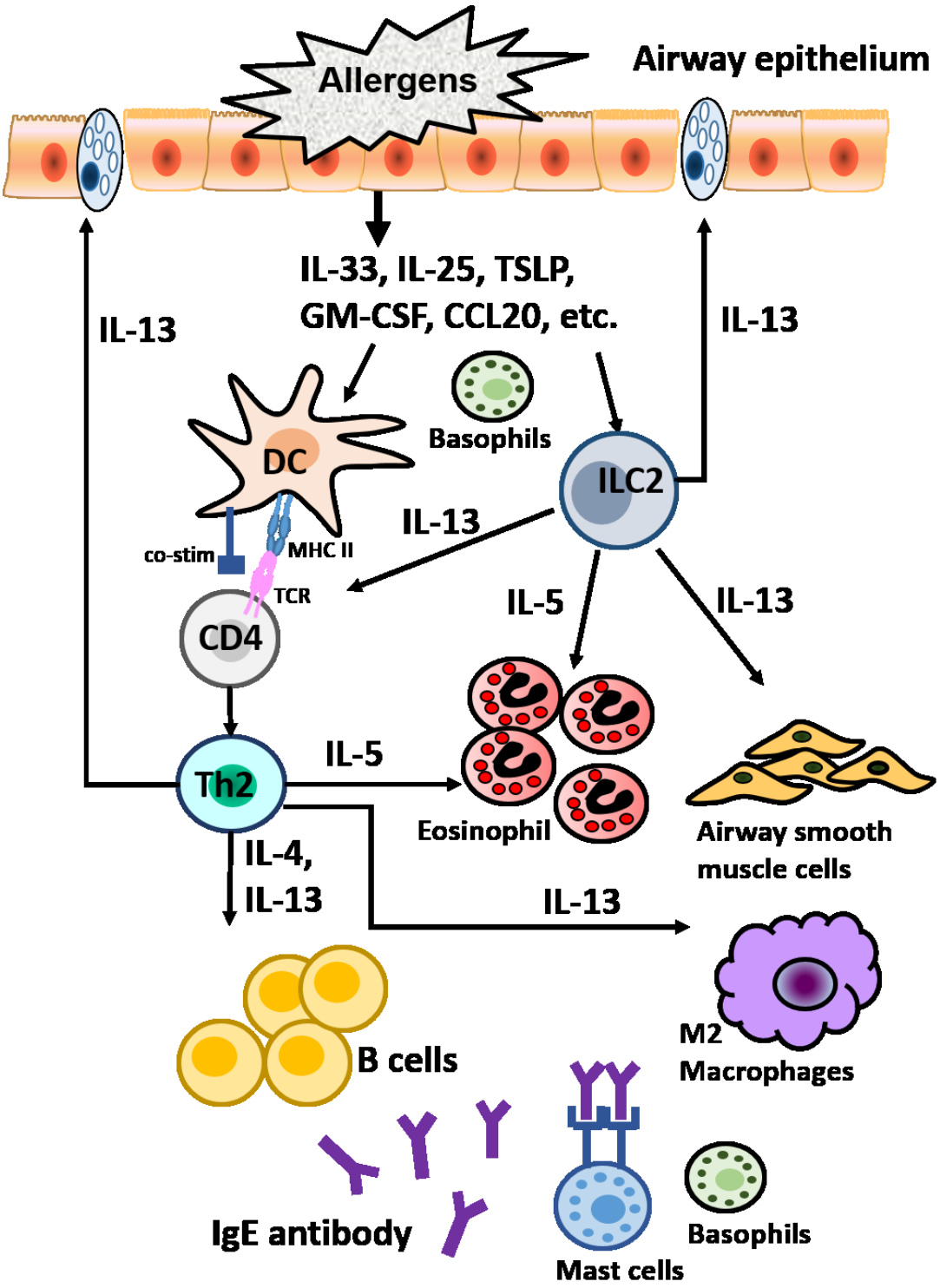
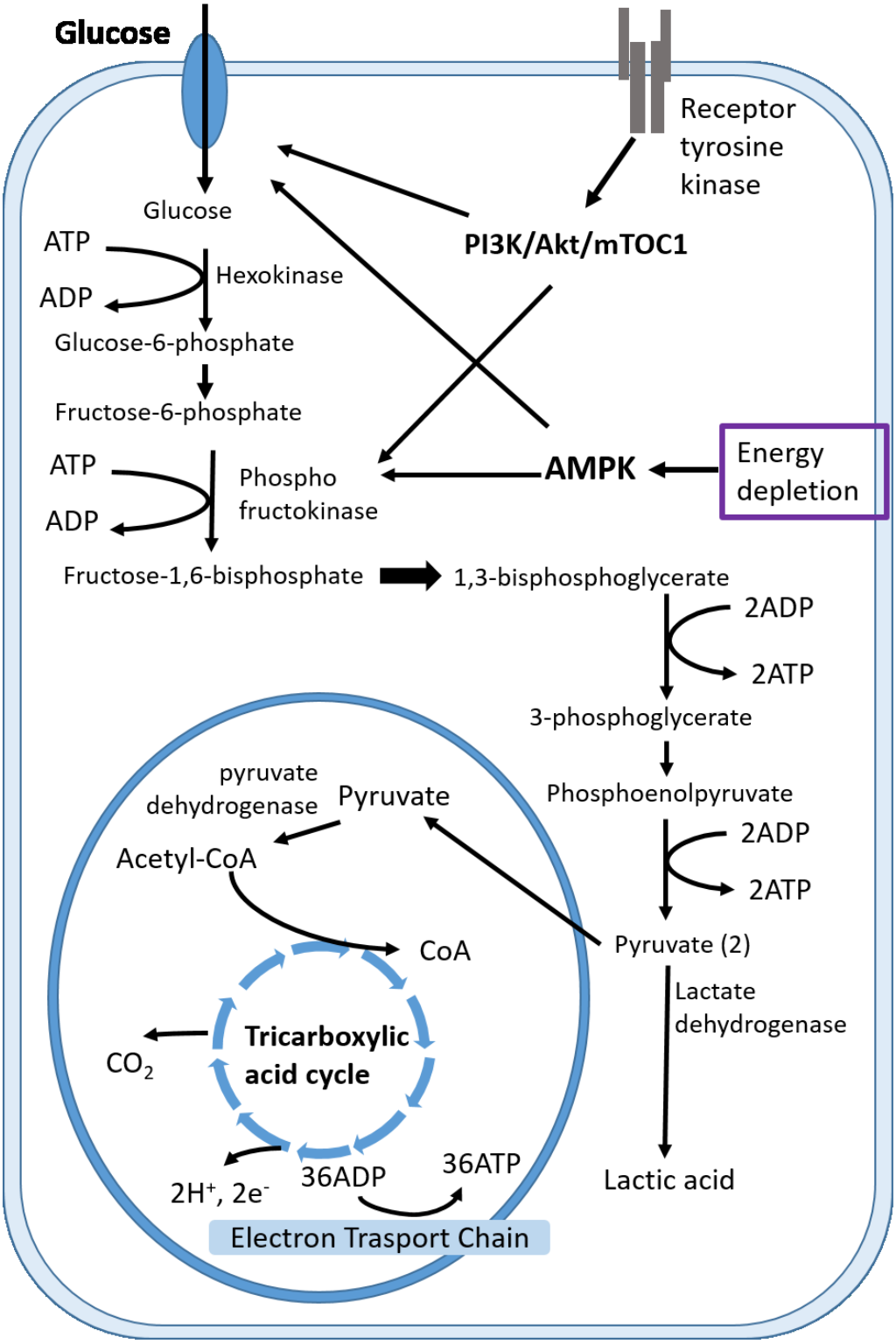


Figure 2: Glucose catabolism overview with a focus on steps involving ATP and the regulation by mTOR and AMPK. When glucose enters a cell from the bloodstream, it is immediately converted to glucose 6-phosphate by hexokinase (HK) and trapped within the cells for subsequent catabolism. It is then converted to fructose 6-phosphate that is converted to Fructose-1,6-biphosphate by phospho fructokinase (PFK). Both HK and PFK require ATP. Following these steps, the molecule with 6 carbons are then split into two molecules of 3 carbons that are converted to 2 molecules of pyruvate, generating 4 molecules of ATP, resulting in a net production of 2 molecules of ATP. The further reactions of pyruvate depend on metabolic conditions and on the nature of the organism. In normal mammalian tissue cells, pyruvate is converted to either lactic acid in the cytosol by lactate dehydrogenase, or converted to acetyl-CoA by pyruvate dehydrogenase in the mitochondria. Acetyl-CoA undergoes further series of reactions that make up the TCA cycle. Altogether, these acetyl-CoA and acetyl-CoA derived from catabolism of amino acid and fatty acid are oxidized and carbon dioxide is yielded. Moreover, the TCA cycle also provides protons and electrons to the electron transport chain in the mitochondria, facilitating its ATP and water production. The TCA cycle generates 34 molecules of ATP, which together with the 2 molecules generated during glycolysis, providing a total of 36 molecules net production of ATP. Activation of receptor tyrosine kinase leads to activation of the PI3K/Akt/mTOC1 signaling cascade that enhances the activity of the glucose transporter, HK and PFK, whereas energy deprivation activates AMPK and promotes the activities of these molecules.

Figure 2: Overview of glucose catabolism with a focus on steps involving ATP and the regulation by mTOR and AMPK.



Chapter 2: Materials and Methods

2.1 Epithelial cell cultures

2.1.1 BEAS-2B and NHBE cells

Transformed BEAS-2B human bronchial epithelial cells were purchased from ATCC and normal human bronchial epithelial cells (NHBE) were purchased from Lonza. Cells were maintained in bronchial epithelial cell growth media (BEGM, Lonza) supplemented with bovine pituitary extract (BPE), hydrocortisone, h-EGF, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, and gentamycin/ampicillin (BEGM SingleQuot Kit Lonza). Cells were grown on collagen/fibronectin coated tissue culture flasks. Cells were passaged when 70-80% confluent using 0.05% trypsin. For experiments, cells were plated at 20,000 cells/well and grown until confluent. Prior to stimulations, cells were starved by culturing them overnight in BEGM devoid of bovine pituitary extract.

2.1.2 16HBE cells

16HBE human bronchial epithelial cells (California Pacific Medical Center, San Francisco, CA) were maintained in Dubeco's minimum essential medium (DMEM, Corning) supplemented with 10% fetal bovine serum (FBS), 2 mmol L glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (c-DMEM). Cells were grown on collagen/fibronectin-coated tissue culture flasks and passaged by means of trypsinization when 80-90% confluent (0.05% Trypsin-EDTA, Gibco). For experiments, cells were plated at 50,000 cells/well and grown until confluent. Prior

to stimulations, cultures were serum starved overnight with DMEM supplemented with 0.1% fetal bovine serum (FBS), 2mmol L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

2.2 Allergen extracts and inhibitors

2.2.1 Allergen extracts

HDM extract, peanut extract, *Alternaria alternata* (*Alternaria*) cell filtrate and cockroach extract were all ordered from Greer and re-suspended in PBS at 2.5 mg/mL based on protein content. Cells were stimulated with HDM (100 μg/mL), *Alternaria* (25 μg/mL), cockroach (25 μg/mL) and peanut (100 μg/mL) for 2 hours.

2.2.2 Inhibitors

In experiment using inhibitors, cells were pretreated with DCA (Sigma), oxamate (Sigma), lactic acid (Sigma) for 1 hour before allergen stimulation, or dimethyloxalylglycine (DMOG) (1 mM, Sigma) for approximately 16 hours before allergen treatment, or cycloheximide (0.5 μM, Santa Cruz), NSC23766 (Rac1i, Tocris), EDTA (0.5 mM, Gibco), Exo1 (20μM, Tocris), oligomycin (4 μM, Tocris), compound C (AMPKi, Calbiochem), Pepinh-MYD (10 μM, Invivogen), Pepinh-Control (10 μM, Invivogen), BX795 (5 μM, Tocris), ATP (50 μM, Sigma), Suramin (100 μM, Sigma) and WRW4 (20 μM, Tocris) for 30 minutes before allergen exposure. Experiments were performed with either H_2O or DMSO controls according to the solvent used for specific inhibitors. In the energy source exchange experiment, cells were cultured with Dulbecco's Modified Eagle's medium (DMEM) (Sigma)

supplemented with 2.5 mM pyruvate (Thermo Fisher Scientific) for approximately 16 hours before allergen treatment.

2.3 Cell metabolism measurement

Live cell respiration was measured using XF extracellular flux analyzer (Seahorse Bioscience). Cell respiration was quantified by oxygen consumption rate (OCR). Glycolysis was measured by extracellular flux acidification rate (ECAR). Specifically, BEAS-2Bs were seeded at 12,000 cells/well (50% confluent) in Seahorse specialized cell culture plate (Seahorse Bioscience), attached and grew overnight. On the next day, cells were washed and switched to buffer free DMEM (Seahorse Bioscience) supplemented with BEGM SingleQuot Kit and calibrated for 1 hour in a CO_2 free incubator. Allergen extracts and inhibitors were prepared and loaded into the Seahorse sensor cartridge (Seahorse Bioscience) at 20 μL per injection port. The concentrations were adjusted so that final concentrations after injection into cells were consistent with the other assays. Then cells were loaded into the analyzer and calibration was performed per the manufacture's manual. After a stable baseline respiration was achieved, OCR and ECAR were measured every 7 minutes for 70 minutes. For lactate secretion, cell culture supernatants or BAL were collected and lactate concentration in them was determined with colorimetric assay (BioVision). For glucose uptake, 10 μL supernatant was taken from each well before and after the allergen and inhibitor treatment. Glucose concentration in them was measured using the colorimetric assay (BioVision) and the reduction of glucose was calculated to reflect the difference of glucose uptake

between conditions. Extracellular ATP levels were determined using the ATP determination kit (Invitrogen) following manufacture's manual.

2.4 Cell viability

Cell viability was assessed for BEAS-2B cells as the live cell numbers post the treatment with inhibitors. In each condition after allergen and inhibitor treatment, live cell numbers were determined using trypan blue or Cell Counting Kit – 8 (Sigma).

2.5 IL-33 subcellular translocation measurement

BEAS-2B cells were treated as described with media or 20 mM DCA for one hour followed by 15 minutes with media or HDM (100 μg/mL). Then proteins from cytosolic, nuclear and cytoskeleton-attached compartments were extracted using the subcellular fractionation kit (Pierce) following the manufacture's manual. IL-33 in the supernatant and various subcellular fractions was measured by ELISA.

2.6 siRNA transfection

Rac1 and AMPK siRNA tranfection was conducted to validate the effects of inhibitors. BEAS-2Bs were plated at 15,000 cells/well in flat-bottom 96-well plates and allowed to attach and grow overnight. The next day, cells were washed 3× with 100 μL sterile PBS. DharmaFECT 1 (Dharmacon) was diluted in BEGM media (Lonza) without supplements at 2 μL/well. In separate eppendorf tubes, scrambled and Rac1 or AMPK siRNA (Dharmacon) were diluted to 5 μM. DharmaFECT 1 and

siRNA dilutions were incubated for 5 minutes at room temperature. They were then combined and the mixtures were incubated for 20 minutes at room temperature before addition to cultures. Cells were then transfected for 48 hours, starved for approximately 16 hours, followed by media or HDM stimulation as described. Supernatants were harvested and cells were suspended in Trizol for RNA isolation.

2.7 Cell culture supernatant IL-33 Western Blot

2 ml of BEAS-2B culture supernatants after exposure to HDM with and without the presence of DCA were collected and concentrated using Amicon Ultra-0.5 mL Centrifugal Filters (EMD Millipore). The concentrated 20 μL protein solution plus loading dye were boiled for 5 min and resolved on NuPage 12% Bis-Tris gels (Invitrogen). Gels were wet transferred to nitrocellulose membranes (Invitrogen) and then blocked in Odyssey Blocking Buffer (LI-COR Biosciences) overnight at 4 °C. Anti-human IL-33 antibody (R&D) was diluted in Odyssey blocking buffer TO 0.1 μg/mL and incubated overnight at 4 °C with medium agitation. Blots were washed three times in PBST for 10 min and then incubated with IRDye secondary antibody (LI-COR Biosciences) in Odyssey blocking buffer plus 0.5% Tween 20 and incubated with gentle shaking for 1 hour (protected from light), and the blots were scanned and quantified on an Odyssey imaging system.

2.8 Extracellular acidity manipulation

Acetic acid was added into BEGM cell culture media at the concentration of 0.1 μL/mL, 0.2 μL/mL, 0.3 μL/mL and 0.4 μL/mL. The basal and adjusted pH values

of the BEGM media were determined using the Mettler Toledo S220 SevenCompact pH meter (Fisher).

2.9 Mice

For airway responses, WT BALB/c males were ordered from Jackson Laboratories (Bar Harbor, ME) between 6 and 8 weeks of age. All mice were housed in a specific pathogen free facility and used one week after delivery. Mice were provided autoclaved food (Lab diet 5010) and water ad libitum. This study was performed in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of Johns Hopkins Bloomberg School of Public Health (JHSPH). The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of JHSPH (Permit Number MO12H97). All surgery was performed under ketamine/xylazine anesthesia, and all efforts were made to minimize suffering.

2.10 Allergen sensitization and challenge

In early time point experiments, mice received intra-tracheally (i.t.) a single dose of HDM (100 μg/40 μL per mouse) or HDM plus intra-peritoneal (i.p.) injection of DCA (5 mg/50 μL per mouse) at 1 hour before and once every 8 hours after HDM challenge. The mice were sacrificed 18h after HDM treatment. To induce allergic asthma, mice were treated as described above on days 0 and 14 with HDM, or HDM plus DCA at 1 hour before and once every 8 hours after HDM challenge for the first 72 hours after each HDM challenge. The mice were sacrificed on day 17. In the

recombinant IL-33 (rIL-33) experiment, mice received intra-nasally (i.n.) a dose of rIL-33 (0.5 μg/50 μL per mouse, ebioscience) on days 0, 3 and 6 with or without DCA as described, and were sacrificed on day 7. Control mice received PBS (i.t. or i.n.) and H_2O (i.p.) in all experiments. Mice were anesthetized with 100 μL Ketamine/Xylazine (Ketamine: 13.6 mg/mL, Zylazine: 2.12 mg/mL) followed by terminal exsanguination.

2.11 Airway responsiveness measurement

For analysis of AHR, mice were anesthetized by IP administration of Ketamine/Xylazine. Mice were then tracheotomized followed by insertion of an 18-gauge cannula into the trachea. Mice were paralyzed with suxamethonium chloride (3 mg/kg), intubated, and respirated at a rate of 120 breaths per minute with a constant tidal volume (0.2 mL). After a stable baseline was achieved, methylcholine (400 μg/kg) was administered intravenously and dynamic airway pressure (cm H_20 × sec) was monitored for 5 min. Following airway measurement, serum, BAL fluid, and lung tissue were harvested and processed as described below.

2.12 Bronchoalveolar lavage (BAL)

Mice were lavaged by flushing the lungs with 1 ml Hanks buffered salt solution (HBSS, Gibco) using a 1 mL syringes inserted into the cannula put in place during airway measurements (above). Lavage fluids were kept on ice until centrifuging for 5 minutes at 500× g, followed by a 1-minute pulse at 5000× g. Supernatants were removed and the total volume logged for future calculations.

Supernatants were stored in Eppendorf tubes at –80° C. The cell pellet was resuspended in ACK buffer for 3 minutes at room temperature to lyse red blood cells. 1 mL of 10% fetal bovine serum (FBS) in PBS was added and cells were again centrifuged as described. Supernatants were discarded and the pellet re-suspended in 200 mL of 10% FBS in PBS. Total cells were determined by trypan blue. Pellets were then adhered to slides by centrifuging in a Cytospin and allowed to dry. Slides were then processed with Diff-Quick (Siemens, Newark, DE) for differential cell counts.

2.13 Serum collection and IgE measurements

After airway reactivity and BAL collection, the peritoneal cavity was exposed and blood collected with a 1 mL insulin syringe from the inferior vena cava. Blood was then transferred into serum collection tubes (BD microtainer). Blood was allowed to clot at room temperature for 30 minutes, after which tubes were centrifuged for 7 minutes at 10,000 rpm. Serum was removed and stored in 96-well culture supernatant plates at –80° C. Serum IgE ELISAs were then performed (BD Opti IgE kit, BD Biosciences).

2.14 Histology

To determine the structural alterations of the airways, formalin-fixed lung sections were embedded in paraffin wax, cut into 5 μ sections and stained with hematoxylin & eosin (H&E). The slides were examined with a 10× objective and a

minimum of three airway sections per animal were examined. Examiners were blinded.

2.15 Lung tissue collection and flow cytometry

After serum collection, the pleural cavity was opened and whole lungs were excised. One section of the right lung was removed, placed in a cryovial, and stored in liquid nitrogen until transfer to -80° C after experiment completion for RNA isolation. The remainder of the lung tissue was placed on a 70 m cell strainer in a 6-well plate containing 6 mL serum-free Roswell park memorial institute medium (RPMI) containing 0.5 mg/mL Liberase (Roche, Madison, WI) + 0.5 mg/mL DNaseI (Sigma, St. Louis, MO) per well. Tissue was minced and incubated in digestion media for 45 minutes at 37°C. After which, the cell strainer was placed on a 50 mL conical tube and the tissue homogenized using the flat end of a 3 mL syringe. Cell strainers were then rinsed with the media remaining the in 6-well plates, followed by 10 mL complete RPMI (10% FBS + 2mmol L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, c-RPMI). Cell suspensions were centrifuged at 1600 rpm for 6 minutes and cell pellets re-suspended in 3 mL ACK lysis buffer for 4 minutes. Lysing process was stopped by the addition of 10 mL c-RPMI, and cells were again centrifuged at 1600 rpm for 6 minutes. Cell pellets were re-suspended in 1 mL c-RPMI and cell counts determined by trypan blue. Lung single cell suspension was plated in a round-bottom 96-well flat-bottom plate for staining. Cell staining was performed at 4°C following incubation with mouse FcBlock (BD) for 30 min. Data was acquired with an LSRII flow cytometer (BD Biosciences, San Jose, CA) equipped

with lasers tuned to 488nm, 633nm, and 405nm. Spectral overlap was compensated and analyzed using the FACSDiVa software (BD Biosciences).

2.16 T cell flow

Lung cells (0.5×10^6) were stimulated with PMA (100 ng/mL) and ionomycin (1 μg/mL) for approximately 16 hours, then Brefeldin A and monensin (eBioscience) were added for 4 hours. Cells were fixed, permeabilized and stained with anti-CD4-PE-Cy7 (RM4–5), anti-TCRβ FITC (H57-597), and anti-IL-13e660 (eBio13A).

2.17 ILC flow

Lung-single cell suspensions were plated at a density of 5×10^6 cells in c-RPMI in a 6-well-flat bottom culture plate. Cells were stimulated with PMA (100 ng/mL) and ionomycin (1 μg/mL) for approximately 16 hours, then Brefeldin A and monensin (eBioscience) were added for 4 hours. ILC2s (Lineage-, CD45+, ST-2+, IL-13+) were identified using following antibodies. Lineage (Lin) cocktail (PerCPCy5.5) contains anti-CD3ε (145-2C11), anti-TCRβ (H57-597), anti-CD11b (M1/70), anti-CD11c (N418), anti-Gr1 (RB6-8C5), anti-B220 (RA3-6B2), and anti-NK1.1 (PK136). Lin- cells were then gated using anti-CD45 (30-F11), anti-ST-2 FITC (T1/ST2) and anti-IL-13 PE (ebio13A).

2.18 Cytokine ELISAs

IL-33, IL-8, and IL-6 levels were measured from BEAS-2B culture supernatants. IL-33 and IL-13 level were measured from BAL of mice. All the cytokine measurements were conducted with ELISA Duo-Sets (R&D).

2.19 Quantitative real-time PCR (RT-PCR)

Total RNA from mouse lungs and epithelial cell cultures was isolated with Trizol according to manufacture's protocol (Life Technologies). RNA was quantified by absorbance at 260 nm and purity determined by 260/280 ratios as measured by NanoDrop. 1 μg of RNA (lung tissue) or 500 ng RNA (epithelial cells) was used as a template for cDNA synthesis. RT-PCR was performed with iTag Universal Syber Green Mix, Hot-Start (BioRad) and specific primers.

2.20 Statistical analysis.

Analysis of variance (ANOVA) followed by the Dunn's post test was used for analysis of differences among multiple groups. Student's t-test was used for comparisons between two groups. P values of less than 0.05 were considered significant.

Chapter 3: HDM-induced Rapid Increase of Anaerobic Glycolysis Mediates IL-33 Release from Bronchial Epithelial Cells

3.1 Introduction

Allergic asthma is a chronic inflammatory disease of the airways that is thought to arise as a result of aberrant type-2 immune responses to innocuous environmental allergens. Of the allergens, house dust mite (HDM) is the most common aeroallergen to which atopic asthmatics are sensitized [375], and sensitization to HDM has been shown to be a major risk factor for the development of asthma [30, 376]. As a result, dissecting the pathways by which HDM induces allergic asthma remains a major goal in asthma research. The pathogenesis of allergic asthma has been extensively studied, and the mechanisms as well as factors involved in driving the downstream pathology of the disease such as eosinophilia, CD4+ Th2 cell infiltration, and bronchoconstriction are well described. However, the initiation of allergic asthma remains to be defined.

It has long been appreciated that airway epithelial cells play critical roles in the initiation of innate and adaptive immune responses to diverse environmental allergens [377, 378], contributing to the development of allergic asthma [227]. The predominant mechanism by which the airway epithelium mediates allergic disease initiation and/or progression is by releasing various cytokines that promote type-2 immune responses [379]. Among these cytokines, the IL-1 like protein IL-33, which has a β-trefoil fold in its carboxy-terminus similar to other IL-1 family members for its binding to the orphan IL-1 receptor (IL-1R) family member ST2, has been shown

to play a central role in allergic inflammation [238]. IL-33 ligation of ST2 has been shown to activate Myd88-dependent signaling pathways in target cells expressing the ST2/IL-1RAcP receptor complex [238, 239] including ILC2s, mast cells, basophils, eosinophils and Th2 cells [380], leading to their production of pro-inflammatory mediators. IL-33 is constitutively expressed and located mainly in the nuclei of tissue structural cells including epithelial cells, endothelial cells and fibroblasts [240]. It was originally thought to function as an "alarmin" as it is released during cell necrosis, while it is cleaved and inactivated by caspases during apoptosis [240-242]. It was also found to be cleaved by calpain as well as neutrophil elastase and cathepsin G to generate mature forms from the full-length protein with enhanced biological activity [243]. More recently, it has been recognized that IL-33 is also released upon environmental exposures to allergens, virus infection and mechanical stress [244-247].

Numerous lines of evidence suggest that IL-33 plays a crucial role in the pathogenesis of allergic asthma. The observation that increased expression of IL-33 in the lung tissue of asthmatic patients [381] as well as the identification of *Il33* and *ST2/Il1rl1* (IL-33 receptor) genes as major asthma susceptibility loci [18] indicate its importance in mediating allergic asthma in humans. Meanwhile, a growing number of studies in experimental models of asthma have demonstrated its critical role in the development of allergen-induced airway hyperresponsiveness, eosinophilia and excessive mucus production [240, 382]. It is believed to drive initial activation of both innate and adaptive aspects of type-2 immune responses via crosstalk (through direct binding of its receptor ST2) with multiple immune cells.

For example, IL-33 contributes to DC activation and migration [255]. It also supports macrophage transition to the M2 subset and amplifies the M2 responses [261]. It promotes mast cell and granulocyte activation as well as pro-inflammatory cytokine release [266, 278]. Most importantly, IL-33 induces IL-5 and IL-13 production by ILC2s and CD4+ T cells [383]. As a result, IL-33 in the lung is always linked to inappropriate activation of immune responses against environmental allergens and local allergic inflammation.

Despite the importance of IL-33 to allergic disease, the mechanisms by which allergens drive its secretion remain incompletely understood. As discussed earlier, it was initially thought to be released as a result of cellular damage/injury and death for its main location in the nucleus [248]. However, several groups have recently proved that allergens and environmental stimuli can also induce live cell release of pre-formed IL-33 within minutes from vesicular compartments other than the nucleus where this cytokine resides [244, 245, 247]. For example, the fungal allergen, *Alternaria alternata* (*Alternaria*) has been shown to induce IL-33 release from airway basal cells without signs of cell death [244] and to be associated with IL-33 release and allergic inflammation [249]. Specifically, Kouzaki et al. report that *Alternaria* induces the rapid release of the danger signal, ATP, by airway epithelial cells. This ATP then activates the P2 purinergic receptor to induce increases in intracellular calcium that are required for IL-33 release [244]. A more recent study supports this hypothesis and demonstrates that the elevated intracellular calcium may activate NADPH oxidase dual oxidase 1 (DUOX1)-mediated activation of epithelial epidermal growth factor receptor (EGFR) and the protease calpain-2 to

process IL-33 into its mature form for release [245]. These studies greatly advanced our understanding of the rapid secretion of IL-33 by airway epithelial cells, but the exact pathway directly facilitating the release of IL-33 was not addressed. However, another group found that release of IL-33 from live cells was dependent on cytoskeleton and cytosolic vesicles-mediated translocation [247].

As the exact pathway mediating IL-33 release is unknown, the involvement of ATP and cytoskeleton suggests that IL-33 live cell secretion is potentially dependent on appropriate energy or substrates generated in the cells during metabolism. In fact, emerging evidence also suggests that cellular glucose metabolism regulates many vital pathways for the release of certain factors such as lipid mediators, cytokines, and hormones during the activation of immune cells [323-326]. IL-33 release from nuclear and vesicular compartments of live cells upon environmental exposure [244, 247] shares many similarities with the release of other mediators regulated by cell metabolism: they are all released as pre-formed substrates from cells, and membrane formed vesicular structures are likely involved. Moreover, recent studies also show that pulmonary resident cells may undergo or be affected by metabolic alterations during allergic asthma [369-372]. For example, it has been shown that OVA sensitized rats have significantly increased expression of proteins related to glycolysis (pyruvate kinase, phosphoglycerate kinase 1, glyceraldehyde 3-phosphate dehydrogenase), calcium signaling, and mitochondrial activities in the lung [371]. OVA-sensitized mice have more lactate in the BAL [372]. Importantly, increased serum lactate levels are observed in humans, which together with the decreased blood pH are associated with asthma severity in humans [369,

370]. Collectively, these studies suggest that an alteration in the balance of glucose metabolism between glycolysis (which generates lactate and acidic environment) and OXPHOS (which consumes oxygen and leads to terminal metabolism of glucose into CO_2 and H_2O) is associated with allergic asthma in both experimental asthma models and human asthmatics.

As the association of glucose metabolism with allergic asthma is more compelling, we hypothesize that HDM-driven IL-33 release may be facilitated by allergen-induced cell glucose metabolism changes in airway epithelial cells. To test this hypothesis, we evaluated the IL-33 release pattern and metabolism phenotype (live cell respiration and glycolytic flux, lactate production, glucose uptake) in the human transformed airway epithelial cell line, BEAS-2B, following treatment with HDM and blockade modulation of the glycolytic pathways.

In the present study, we observed rapid live cell secretion of IL-33 by HDM independently of *de novo* protein synthesis. This is concomitant with an immediate increase in glycolytic flux. When the enhanced glycolysis was inhibited by DCA, IL-33 release was also reduced. These results confirmed our hypothesis that HDM-induced rapid increase of glycolytic flux associated with IL-33 release in airway epithelial cells.

3.2 Results

3.2.1 HDM-induced rapid live cell release of IL-33 independently of *de novo* protein synthesis.

We first sought to understand the concentration-response relationship between HDM exposure and IL-33 production by airway epithelial cells. To determine the optimal concentration of HDM for the analyses, BEAS-2B cells were seeded at 20,000 cells/well and serum starved overnight. The cells were then treated with HDM at varying concentrations (50 μg/mL, 100 μg/mL and 200 μg/mL) for 2 hours, and IL-33 levels in the supernatants were measured with ELISA. We found that HDM induced significant increases in IL-33 release at all the tested concentrations, with a trend suggesting that the release of IL-33 peaked when at a dose of 100 μg/mL of HDM. At concentrations lower than 100 μg/m, IL-33 levels were around the detection limit of ELISA (data not shown). Moreover, a previous study published by our group found that 100 μg/mL HDM exposure was the optimal concentration for analyzing HDM-induced release of CCL20 [233], a chemokine likely playing important roles in allergic asthma by recruitment of immature DCs through its receptor CCR6 [384]. Taken these data together, we used 100 μg/mL HDM in the subsequent analysis for both technical and biological reasons. To understand the temporal pattern of HDM-induced IL-33 production, BEAS-2B cells were seeded at a density of 20,000 cells/well and then serum starved for 24 hours followed by treatment with either media control or HDM (100 μg/mL) for varying lengths of time (30 minutes, 2 hours, 4 hours, 8 hours and 24 hours) [233, 384]. IL-33, IL-6 and IL-8 were measured by ELISA in the cell culture supernatants. Consistent with previous reports, we found that HDM induced a time-dependent rapid increase in IL-33 release. IL-33 was released as early as 30 minutes and peaked at the same time post-exposure (Fig. 3B). In the contrast, IL-6 and IL-8 were

not observed in the supernatants until 2 hours post-HDM exposure, and peaked at 24 hours after the beginning of HDM treatment (Fig. 3C – D).

To determine whether HDM-induced IL-33 release resulted from cell death, cell viability was determined using WST-8. Specifically, WST-8 is reduced by cellular dehydrogenases to an orange formazan product that is directly proportional to the number of living cells, so live cell number was reflected by the absorbance at 450 nm after a 2 hour incubation with WST-8. The viability of cells didn't significantly differ between cells treated with HDM and media (Fig. 3E). To investigate whether the secreted IL-33 was newly synthesized or released pre-formed from an intracellular store, we treated cells with cycloheximide, an inhibitor of eukaryotic cell protein translation [385]. Following the same culture protocol described above, BEAS-2B cells were pre-treated with 0.5 μM cycloheximide for 30 minutes followed by HDM (100 μg/mL) treatment for 5, 10, 50 and 100 minutes. As expected, HDM exposure induced the rapid release of IL-33, and cycloheximide did not inhibit IL-33 secretion (Fig. 3F), indicating that the rapidly secreted IL-33 was from pre-formed intracellular storage of the protein.

3.2.2 HDM exposure caused rapid concomitant increases in glycolysis and IL-33 release in airway epithelial cells.

As discussed above, alterations in glucose metabolism have been associated with the rapid induction of immune cells and cytokine release, and we hypothesize that HDM also induces rapid metabolic alteration, likely increased glycolysis, that is associated with IL-33 secretion. According to the known glucose metabolic

pathways, one molecule of glucose is metabolized to pyruvate, which is then either converted via glycolysis to lactic acid (1 molecule of lactate and 1 proton) in the cytosol, or fully oxidized to CO_2 and H_2O via oxidative phosphorylation (OXPHOS) in the mitochondria in the presence of O_2 [330]. To investigate whether allergen exposure of epithelial cells is associated with changes in cellular glucose metabolism, we measured the production of extracellular protons (extracellular acidification), oxygen consumption, lactate production and glucose uptake in BEAS-2B cells. Specifically, cells were seeded at a density of 12,000 cells/well. They were cultured overnight, and then placed in a closed chamber with controlled air exchange in an extracellular flux analyzer (Seahorse), which monitored culture media pH and calculated the glycolytic flux by extracellular acidification rate (ECAR) as well as O_2 partial pressure changes to calculate the rate of OXPHOS as oxygen consumption rate (OCR). Cells were measured every 7 minutes for a total of 70 minutes. Once the baseline respiration rate was obtained after the first 3 measurements, media or HDM (final concentration of 100 μg/mL) was injected into the cell chamber before the 4^{th} measurement. Compared to cells injected with media, there was a significant immediate increase in glycolysis (ECAR) in HDM injected cells (Fig. 4A), while OXPHOS remained relatively stable (Fig. 4B). To determine whether similar changes were observed in lactate production, lactate levels in the culture supernatants of BEAS-2B cells treated with HDM were measured by colorimetric assays. Similarly, glucose levels in the HDM-treated cell supernatants were calculated to reflect glucose uptake. Consistent with the ECAR and OCR changes we observed with HDM treatment, lactate production was

significantly increased after 2 hours of HDM exposure as compared to that measured in media-treated cells (Fig. 4C). Likewise, the calculated glucose uptake was also significantly increased in cells exposed to HDM versus those treated with media (Fig. 4D). The concomitantly conducted ELISA revealed that cells treated with HDM secreted IL-33, while controls cells did not (Fig. 4E). To determine whether our findings in the transformed epithelial cell line (BEAS-2B cells) translated to primary human epithelial cells, normal primary human airway epithelial cells (NHBE) were seeded at 20,000 cells/well and treated with either media or HDM (100 μg/mL) for 2 hours. Consistent with our findings in BEAS-2B cells, we observed that HDM exposure resulted in significantly increased lactate production and IL-33 release by NHBE cells (Fig. 4G).

To determine whether these metabolic changes were unique to HDM or were a common feature of allergenic stimuli, we compared the effects of HDM (100 μg/mL), LPS (500 ng/mL), peanut extract (100 μg/mL), *Alternaria* (25 μg/mL) and cockroach (25 μg/mL) extracts on extracellular lactate levels. We observed that while HDM exposure induced significant increases in lactate production as compared to media controls, none of the other stimuli induced lactate production in airway epithelial cells (Fig. 4F). Taken together, our results showed that HDM exposure caused rapid increases in glycolytic flux, concomitant with IL-33 release. Whether these two events are linked is currently unknown.

3.2.3 Inhibition of anaerobic glycolysis diminished HDM-induced IL-33 secretion by airway epithelial cells.

Given that we observed significant increases in glycolysis along with IL-33 release from airway epithelial cells over the same timeframe, we hypothesized that the two events are linked and that changes in glycolysis may regulate IL-33 release from airway epithelial cells. To assess the effect of blockade of glycolysis on IL-33 release, we utilized sodium dichloroacetate (DCA), a small molecule inhibitor of pyruvate dehydrogenase kinase which increases glucose oxidation by inhibiting mitochondrial pyruvate dehydrogenase kinase 1 (PDK1), thereby decreasing lactate production and glycolytic flux [386]. Specifically, we determined the effects of DCA on lactate and IL-33 levels in HDM-exposed cells. To determine the concentration of DCA that modulated lactate without causing cellular toxicity, BEAS-2B cells were seeded at 20,000 cells/well and treated with varying concentrations of DCA (0.2 mM, 2 mM, and 20 mM) for 1 hour. Lactate levels in the supernatants were measured. It was observed that as the concentration of DCA increased, the level of lactate in the media was decreased (Fig. 5A). To determine whether DCA was toxic to these cells, the cells were treated with the highest concentration of DCA at 20 mM for 4 days, and cell viability was determined everyday at the same time using the WST-8 assay. We confirmed that DCA treatment had no cytotoxic effects, as the viability of cells treated with DCA did not differ from those in media alone (Fig. 5B). Based on these data, we utilized 20 mM of DCA to pre-treat HDM- and media-treated BEAS-2B cell 1 hour prior to HDM exposure, and conducted the same assays of cellular metabolism and IL-33 measurement as described in 3.2.2.

As shown in Fig. 5C, DCA inhibits PDK1, a natural regulator of OXPHOS, to promote mitochondrial activity and leads to inhibition of anaerobic glycolysis. For

the Seahorse experiments, cells were seeded and treated with 20 mM DCA for 1 hour before loading onto Seahorse for ECAR and OCR measurements. Again, we saw a significant increase in ECAR immediately after HDM (100 μg/mL) injection into the cell culture. However, cells pre-treated with DCA had significantly lower HDM-induced ECAR (Fig. 5D) due to a shift of glucose metabolism pathways towards OXPHOS (Fig. 5E). To determine whether lactate, glucose and IL-33 levels changed in concert with the ECAR and OCR changes, these mediators were measured in BEAS-2B cells pre-treated with either DCA or media, following by media or HDM (100 μg/mL) treatment for 2 hours. DCA treatment of cells significantly reduced the HDM-induced increases in lactate production (Fig. 5F), glucose uptake (Fig. 5G) and IL-33 release (Fig. 5H). To confirm that DCA did not cause cell death in the presence of HDM, the viability of these cells was determined by trypan blue exclusion. DCA treatment did not cause cell death in the presence of HDM (Fig. 5I). As the inhibition of anaerobic glycolysis with DCA significantly reversed allergen-stimulated increases in glycolytic flux and IL-33 release, our results suggest that that the rapid increase of glycolysis by HDM exposure was associated with its induction of IL-33 release by airway epithelial cells.

To confirm that inhibition of anaerobic glycolysis reduced HDM-induced IL-33 release, we modulated glycolysis with another inhibitor oxamate to analyze its effects on IL-33. Similarly, to determine the concentration of oxamate that modulated lactate without causing cellular toxicity, BEAS-2B cells were seeded at 20,000 cells/well and treated with varying concentrations of oxamate (12.5 mM, 25 mM, 50 mM and 100 mM) for 1 hour. Lactate levels in the supernatants revealed

that as the concentration of oxamate increased, lactate production was decreased (Fig. 6A). To determine whether oxamate was toxic to these cells, live cell numbers were determined using trypan blue. We observed no significant cell death after treatment with oxamate (Fig. 6B). Based on these data, we utilized 50 mM of oxamate to pre-treat BEAS-2B cells for 1 hour followed by 2 hours HDM exposure. Similar as DCA treatment, as HDM consistently induced IL-33 release by BEAS-2B cells, oxamate treatment significantly reduced IL-33 in the media (Fig. 6C).

3.3 Discussion

As IL-33 has been shown to be critical in the initiation of allergic inflammation [240], our finding that allergens induce the rapid release of IL-33 from the cells lining the airways through a novel mechanism involving alterations in cellular metabolism represents an important advance in our understanding of the mechanisms by which allergens initiate the inflammatory responses associated with the asthma phenotype. Specifically, we showed that HDM exposure led to early IL-33 release from airway epithelial cells within 30 minutes (Fig. 3B), and confirmed that this release is independent of cell viability changes (Fig. 3E). Unlike the previous hypothesis that IL-33 is mainly released from the damaged nucleus upon cell necrosis, our results have provided additional support for the contention that IL-33 can be released from live cells in response to external stimuli such as certain allergens [244-247]. One potential limitation of the current study is the lack of a comprehensive concentration-response analysis of HDM-induced IL-33. In the recently published study showing that HDM induced rapid release of IL-33, the

authors showed that the levels of IL-33 released by primary human cells were increased with greater doses of HDM (within 0 – 30 μg/mL), and cells from asthmatic individuals produced more IL-33 compared to those from healthy individuals [245]. In our studies, we used a higher concentration at which HDM could induce significant release of both IL-33 and CCL20, the important epithelial-derived cytokines playing roles in asthma, as we intent to mimic the possible condition in the airway in which multiple cytokines can be induced by environmental stimuli [387]. Moreover, in a theoretical analysis of the physiological relevant concentrations of HDM in the airways, it was acknowledged that while no direct evidence was available to translate the epidemiological measurements of HDM levels in the ambient into concentrations for *in vitro* analysis, normal exposure could lead to very high concentrations of HDM allergens depending on the closeness of individuals to dust-rich areas, accumulative exposures, and the anatomical conditions of the airways. As a result, it was concluded that physiological relevant concentrations of HDM could range from nanogram level to milligram level [28].

The fact that the IL-33 release is independent of *de novo* protein synthesis (Fig. 3F) suggests a mechanism of rapid translocation of pre-formed proteins from stores in the cell to the extracellular milieu. This is consistent with a recent report showing that HDM exposure induces the early release of IL-33 independently of changes in message expression [245]. Taken together our studies have confirmed the new concept that IL-33 is secreted by live cells under stress, and provide a potential mechanism for the link between HDM exposure and allergic asthma. Moreover, in comparison to the distinct release pattern of IL-6 and IL-8 by airway

epithelial cells, the current data highlights the uniqueness of IL-33 secretion induced by HDM, suggesting the importance of understanding its regulatory mechanisms.

Our study has provided several lines of evidence to support a novel mechanism by which dust mite exposure at the airway surface triggers the release of the type-2 promoting cytokine, IL-33, namely through induction of changes in cellular metabolism, resulting in a shift towards anaerobic glycolysis for energy production. Indeed, we analyzed the metabolic phenotype of airway epithelial cells post-HDM exposure and reported for the first that HDM caused a rapid increase in glycolysis in airway epithelial cells. Using the advanced extracellular flux analyzer, we showed that HDM exposure led to a rapid increase in glycolysis within minutes (Fig. 4A), and confirmed this finding with increased lactate production (Fig. 4C) and glucose uptake (Fig. 4D) by the cells. Normally, after cells take up glucose molecules, they will first go through a few steps to convert one molecule of glucose into two molecules of pyruvate. Following this, a large portion of the pyruvate will be metabolized into Acetyl-CoA, the substrate for the TCA cycle that occurs in the mitochondria (OXPHOS), and the rest of the pyruvate will remain in the cytosol to be converted into lactic acid (lactate and proton) [327]. Thus, under normal physiological conditions, glycolysis and OXPHOS are balanced. However, although HDM induced significant increases in the amount of glucose taken up by the cells, instead of both glycolysis and OXPHOS being increased, only glycolysis was significantly induced, while the OXPHOS remained relatively stable (Fig. 4B). We thus concluded that HDM exposure of the cells might cause an imbalance between

glycolysis and OXPHOS, resulting in increased glycolytic flux. This tenet is supported by our observation that HDM exposure resulted in elevations in the end product of glycolysis namely lactate, as well as an increased demand for its substrate, glucose. However, this finding is restricted to the earlier phase (within 2 hours) post-HDM exposure. Although IL-33 release peaks at the earlier time point, the live cell respiration data also suggested that HDM-induced glycolytic flux persisted for at least 70 minutes (the maximum length of time that cells could be maintained under the experimental conditions required for Seahorse measurements) (Fig. 4A). These data pose new questions for future investigation: do HDM-induced early metabolic alterations persist for longer than 70 minutes? Does HDM induce different metabolic phenotypes in cells after different lengths of exposure? Whether a later (maybe also different) metabolic phenotype is associated with IL-33 release? Do metabolic alterations contribute to epithelial response to HDM exposure via mechanisms other than mediating IL-33 release? Answering these questions will provide insights into HDM-induced physiological and pathological changes in the airway epithelium.

Although this is the first report showing that HDM exposure induces metabolic changes in airway epithelial cells, our finding is consistent with previous reports of altered metabolism in asthma patients, which may contribute to the acidic airway lining fluids observed in asthmatic individuals [369, 370]. Moreover, previous reports of alterations in lung glycolytic protein profiles following OVA exposure of rats [371] further supports our hypothesis that cellular metabolic

alterations may represent an early response of cells to environmental stimuli and accounts for allergic inflammation.

The concept of exogenous stimuli causing cellular metabolic changes has recently garnered considerable interest. Indeed, activation of a wide variety of immune cells including granulocytes [333-336], DCs [337, 338], macrophages [339, 340], T cells [321, 341, 342] and B cells [343, 344] has been shown to be dependent on metabolic alterations. Specifically, a recent study reported that LPS exposure caused rapid induction of glycolytic activity in DCs that drove cytokine production and MHC molecule loading [338]. Although this observation might imply that LPS and HDM may activate similar signaling pathways leading to IL-33 release and glycolytic flux, both our colleagues (data not published) and a recent study have shown that LPS exposure does not induce early IL-33 release from airway epithelial cells [245]. Moreover, by measuring lactate production of these cells following LPS exposure, we showed that LPS did not induce increased glycolytic flux (Fig. 4F). Although the BEAS-2B cells do express the TLR4, which recognizes LPS [388], it is reasonable to hypothesize that TLR4 activation may preferentially lead to different downstream signaling pathways in epithelial cells compared to DCs. Also, it is possible that other portions of HDM regulate the induction of IL-33 and glycolysis.

Interestingly, our findings further suggest that the induction of IL-33 through stimulation of glycolysis may be somewhat specific to HDM. In addition to LPS, other allergens and contaminants of allergens including peanut, *Alternaria* and cockroach allergens did not increase lactate production (Fig. 4F). This is surprising since *Alternaria* [244, 245], peanut (data not published) and cockroach [389] can

induce IL-33 release from airway epithelial cells. Although these results may appear discordant, it is possible that either certain unique component(s) of HDM drive the induction of glycolysis, or that IL-33 release by airway epithelial cells is regulated by multiple pathways depending upon the nature of the stimulus. Evidence supporting such a hypothesis is lacking, as little research has been done comparing the IL-33 induction capacity of different components from each allergen extract. Likewise, none of the studies to date have elucidated the secretion pathways of IL-33 following allergen exposure besides showing the involvement of calcium signaling [244, 245]. Understanding the underlying pathways for IL-33 release, or in this case, translocation, would provide some insights. However, the current data should also be interpreted with caution. In our study, concentration-response and viability analyses for the other stimuli have not been conducted. Although BEAS-2B cells were reported to be resistant to LPS toxicity at the tested concentration [390, 391], and *Alternaria* exposure was found to have very low toxic effects on BEAS-2B cells [392], a thorough analysis is still needed to provide a clear answer.

Measuring glycolysis solely as changes in lactate levels also has its limitations. Lactate is only an end product of anaerobic glycolysis. Thus our data does not rule out the possibility that other allergens also induce metabolic alterations under the following circumstances: (1) other allergens induce pathways or mechanisms compensating the production of lactate, shadowing the induction of lactate; (2) other metabolic alterations are induced which also provides energy, signaling substrates, or physiological environment as provided by glycolysis to contribute to IL-33 release; (3) other allergens induce cellular metabolism changes

within a different timeframe, making detection of lactate at this moment difficult. As a result, the current data should not be taken as the final conclusion that only HDM induces metabolic alterations in airway epithelial cells, and further phenotyping of airway epithelial cell metabolism after exposure to other environmental stimuli should be conduction.

Overall, our study is successful in attempting to understand whether environmental allergen causes cell metabolic changes. Our glycolysis blockade studies utilizing the small molecular inhibitor, DCA, further provided a link between allergen-triggered changes in glycolysis and IL-33 release for the first time. Our results were consistent with a previous study showing that DCA treatment of mice abrogates ragweed-induced allergic airway inflammation [26]. More importantly, our studies provide a potential mechanism by which allergen-driven alterations in glycolysis regulate allergic inflammation, namely through the early induction of the type-2 promoting cytokines, IL-33. Moreover, as IL-33 is a pivotal cytokine in type-2 immune responses with unclear regulation mechanism, our novel finding of cell metabolism as a contributor of its release also provides insights into how environmental stimuli induce the secretion of IL-33 to initiate allergic inflammation.

More than providing answers to the scientific questions we are asking, our data also supports the possibility of using DCA for the blockade of IL-33. DCA has been used as a clinically approved oral therapy for lactic acidosis [393-395], and can reverse the Warburg effect in cancer cells to bio-energetically improve prognosis of cancer therapies [396, 397]. A recent study revealed amelioration effect of DCA on ragweed-induced allergic airway inflammation [370], but they did not provide any

description of the effects of DCA on airway epithelial cells. Our study demonstrated that while DCA reversed HDM-induced glycolysis enhancement in airway epithelial cells, it also reduced the release of IL-33. Considering the potential roles of IL-33 in multiple diseases and health conditions, the use of DCA may extend beyond lactic acidosis and cancer.

As we show an association between HDM-induced increases in glycolytic flux and IL-33 release, we took one step further and confirmed this using another glycolysis inhibitor, oxamate (Fig. 6). More than just reproducing our observation, utilizing glycolysis inhibitors targeting different pathways also provides hints to the possible mechanism by which glycolysis is linked to IL-33 release. As discussed earlier, DCA inhibits PDK1 and leads to the enhancement of OXPHOS. The increase in OXPHOS then consumes more pyruvate, the substrate for generating lactate via anaerobic glycolysis, and leads to reduced glycolysis activities [398]. Unlike DCA, oxamate directly inhibits lactate dehydrogenase (LDH) and thus inhibits the production of lactic acid, the very last step in anaerobic glycolysis [399]. While both inhibitors block glycolysis, the shared pathway that is blocked is the pyruvate conversion into lactic acid, which generates lactate, protons, and promotes the generation of glycolysis-derived ATP [330]. As both DCA and oxamate reduced HDM-induced IL-33 release, it is thus reasonable to hypothesize that the reduction of IL-33 is associated with reductions in glycolysis-derived lactate, proton, or glycolytic ATP.

However, despite the novel observation that HDM-induced glycolysis is associated with IL-33 release, the current study has certain general technical

limitations. Specifically, the transformed airway epithelial cell line BEAS-2B may not fully represent the response of epithelial cells in the airway. Our data with NHBE cells suggest that HDM exposure also causes concomitant increases in glycolysis and IL-33 release in normal primary airway epithelial cells. Although this observation supports the importance of metabolic changes in allergen-driven IL-33 release in normal cells, studies assessing the metabolic responses of primary airway epithelial cells from asthmatics and healthy controls exposed to HDM is still needed. Both our colleagues and a recently published study revealed that airway epithelial cells isolated from asthmatics produce more IL-33 after HDM exposure compared to cells from normal individuals [245]. Whether glycolysis increases induced by HDM are also greater in asthmatics compared to healthy subjects remains to be determined. Moreover, the current culture system may also limit the generalizability of our results, as they are only 2-dimensional, whereas epithelial cells reside in a 3-dimensional structure in the airway lumen. To better emulate the epithelium *in vivo*, an air-liquid interface culture system that allows cells to differentiate into the multi-cellular layer seen *in vivo* would be utilized for future studies.

Overall, our data strongly supports the hypothesis that HDM induces metabolic changes in airway epithelial cells that are associated with IL-33 secretion. Further studies are needed to elucidate the underlying mechanisms/pathways through which cell metabolism contributes to IL-33 secretion induced by HDM.

3.4 Figures

Figure 3: HDM induced rapid live cell release of IL-33 independently of *de novo* protein synthesis. BEAS-2B cells were seeded in flat-bottom 96-well plates at a density of 20,000 cells/well and then serum starved for 24 hours followed by treatment with either media control or HDM (50 μg/mL, 100 μg/mL, and 200 μg/mL) for 2 hours. (A) IL-33 levels in the supernatant were determined. To understand the temporal pattern pattern of cytokine release induced by HDM, BEAS-2B cells were seeded at a density of 20,000 cells/well in flat-bottom 96-well plates and serum starved overnight. These cells were then treated with either media control or HDM (100 μg/mL). Cell culture supernatants were collected at 30 minutes, 2 hours, 8 hours and 24 hours post-exposure for (B) IL-33, (C) IL-6 and (D) IL-8 measurement. (E) Cell viability was determined at the end of the 2 hours HDM exposure. To determine if de novo protein synthesis was required, (F) cells were seeded and treated with 0.5 μM cycloheximide for 30 minutes and then 100 μg/mL HDM (conducted by Smole) for IL-33 release. Data presented as mean values ± SEM for biological replicates n=3-6, *$p<0.05$, ****$p<0.0001$ (student's t test, one-way ANOVA and Dunn's post test).

Figure 3: HDM induced rapid live cell release of IL-33 independently of *de novo* protein synthesis.

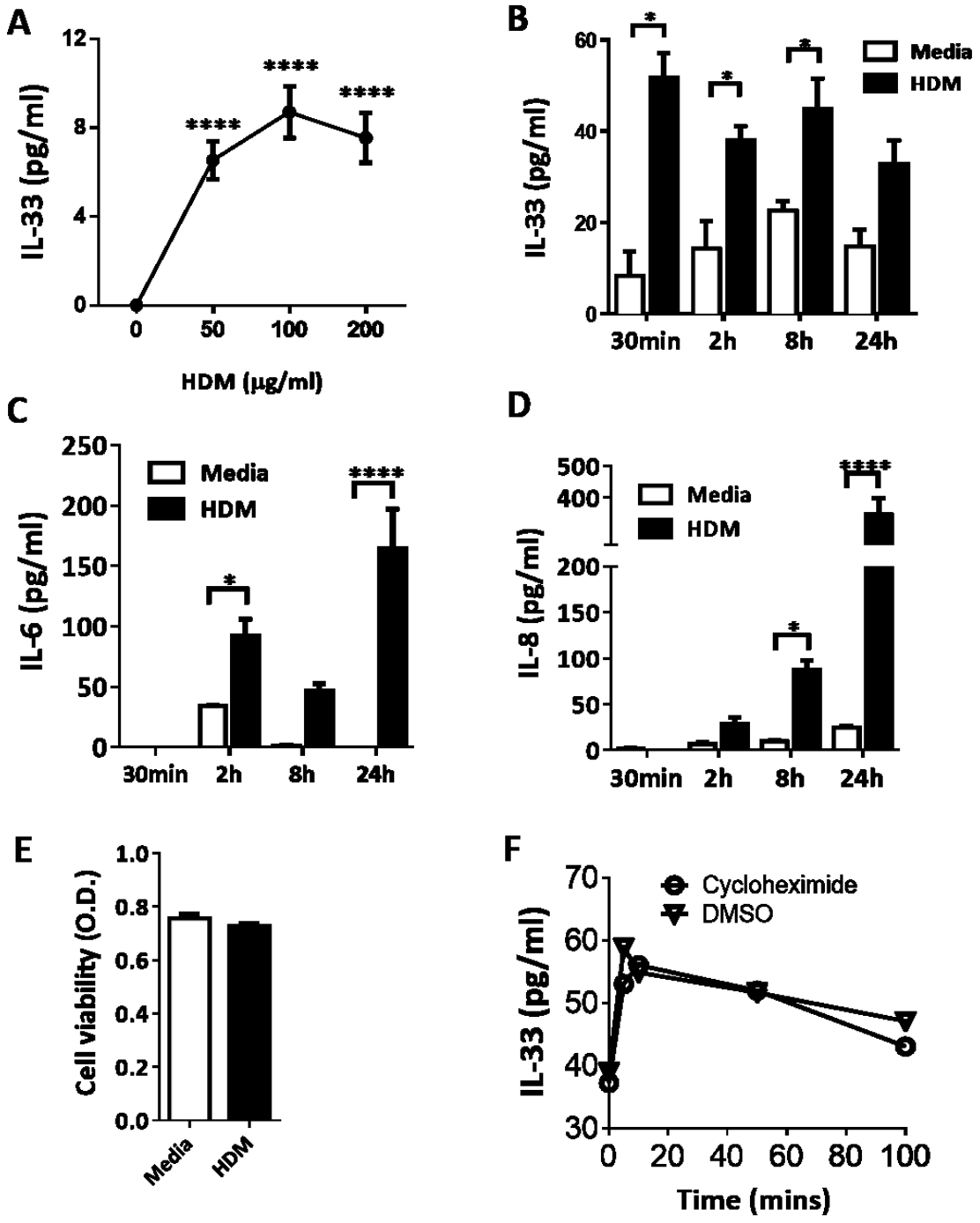


Figure 4: HDM exposure caused rapid concomitant increases in glycolysis and IL-33 release in airway epithelial cells. BEAS-2B cells were seeded in flat-bottom 96-well plates at a density of 12,000 cells/well for live cell respiration measurements (Seahorse), and 20,000 cells/well for lactate production, glucose uptake and IL-33 measurements. For Seahorse assays, cells were equilibrated in a CO_2 free tank with buffer free media for 1 hour and then loaded into the Seahorse. (A) ECAR and (B) OCR were then measured for 10 times within 70 minutes following the manufacturer's instructions, HDM (100 μg/mL final concentration) was injected before the 4^{th} measurement. For lactate, glucose and IL-33 measurements, cells were seeded and then serum starved for 24 hours followed by treatment with either media control or HDM (100 μg/mL) for 2 hours. Cell culture supernatants were collected for (C) lactate, (D) glucose and (E) IL-33 measurement (Chapter 2.12 & 2.13). To test whether other environmental stimuli also induced increase glycolysis, BEAS-2B cells were exposed to HDM (100 μg/mL), LPS (500 ng/mL), peanut (100 μg/mL), *Alternaria* (25 μg/mL) and cockroach (25 μg/mL) for 2 hours, and (F) lactate levels in the supernatants were determined. To validate these data in primary normal airway epithelial cells, NHBE cells were seeded in flat-bottom 96-well plates at a density of 20,000 cells/well, serum starved and treated with either media or HDM (100 μg/mL) for 2 hours. Cell culture supernatants were collected for (G) lactate and IL-33 measurements. Data presented as mean values ± SEM for biological replicates n=3-6, *p<0.05, **p<0.01, ****p<0.0001 (student's t test, one-way ANOVA and Dunn's post test).

Figure 4: HDM exposure caused rapid concomitant increases in glycolysis and IL-33 release in airway epithelial cells.

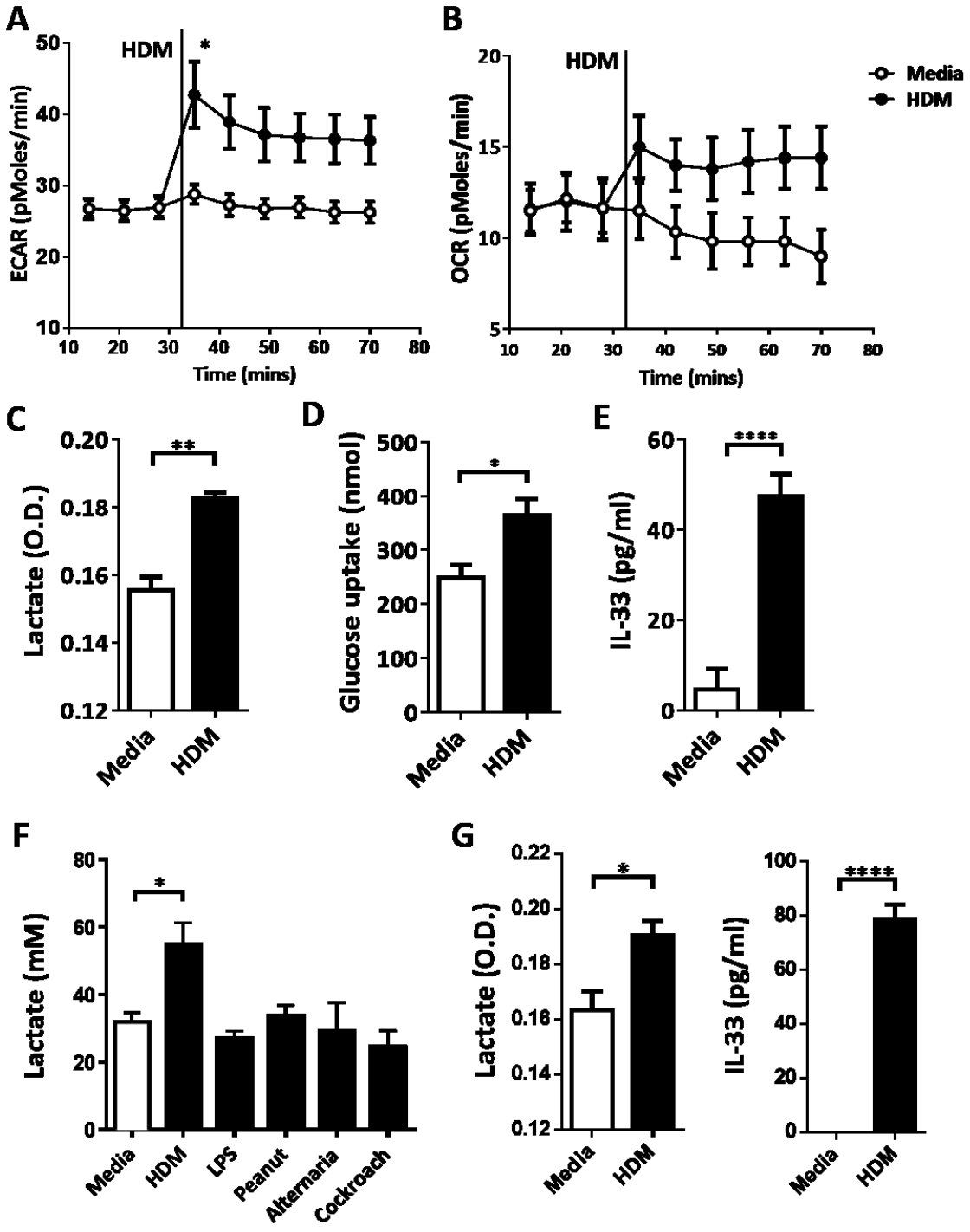


Figure 5: Inhibition of anaerobic glycolysis by DCA diminished HDM-induced IL-33 secretion by airway epithelial cells. To determine the correlation between HDM-induced early IL-33 release and glycolytic flux, BEAS-2B cells were seeded at 20,000 cells/well and treated with DCA (0.2 mM, 2 mM, 20 mM) for 1 hour. (A) Lactate levels in the cell culture supernatants were determined and 20 mM was identified as the effective dose of DCA. The cells were then treated with 20 mM DCA for 4 days, and (B) cell viability was measured with WST-8 every day at the same time. The cells were then seeded as described in Figure 4, pre-treated with 20 mM DCA for 1 hour to (C) inhibit glycolytic flux and then exposed to HDM (100 μg/mL) for measurements of (D) ECAR, (E) OCR, (F) lactate production, (G) glucose uptake and (H) IL-33 release as described in Figure 4. To assess the cell viability changes after DCA treatment in the presence of HDM, cells were pre-treated with either media or 20 mM DCA followed by HDM (100 μg/mL) exposure for 2 hours, (I) and the numbers of live cells were assessed with trypan blue. Data presented as mean values ± SEM for biological replicates n=3-6, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (one-way ANOVA and Dunn's post test).

Figure 5: Inhibition of anaerobic glycolysis by DCA diminished HDM-induced IL-33 secretion by airway epithelial cells.

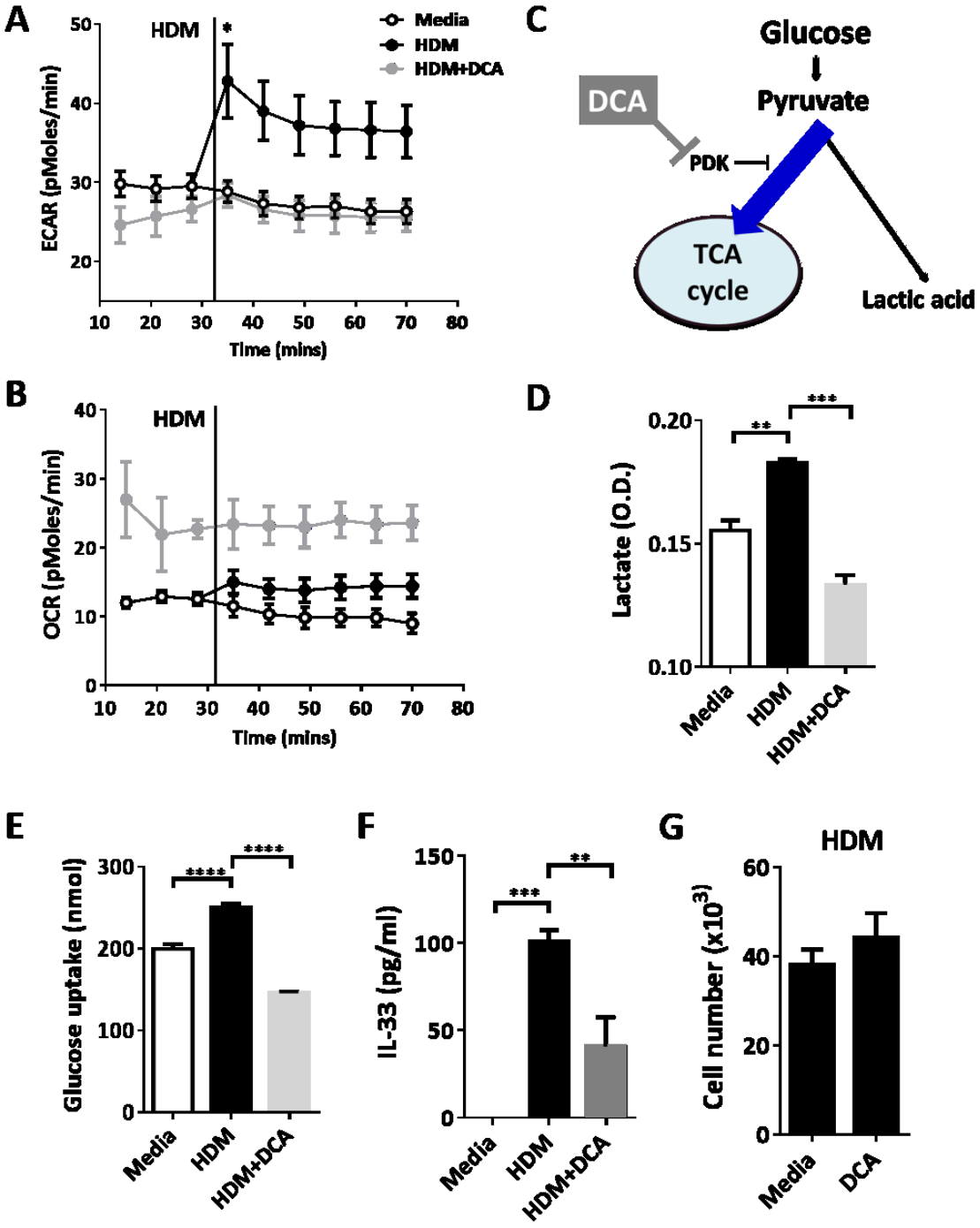
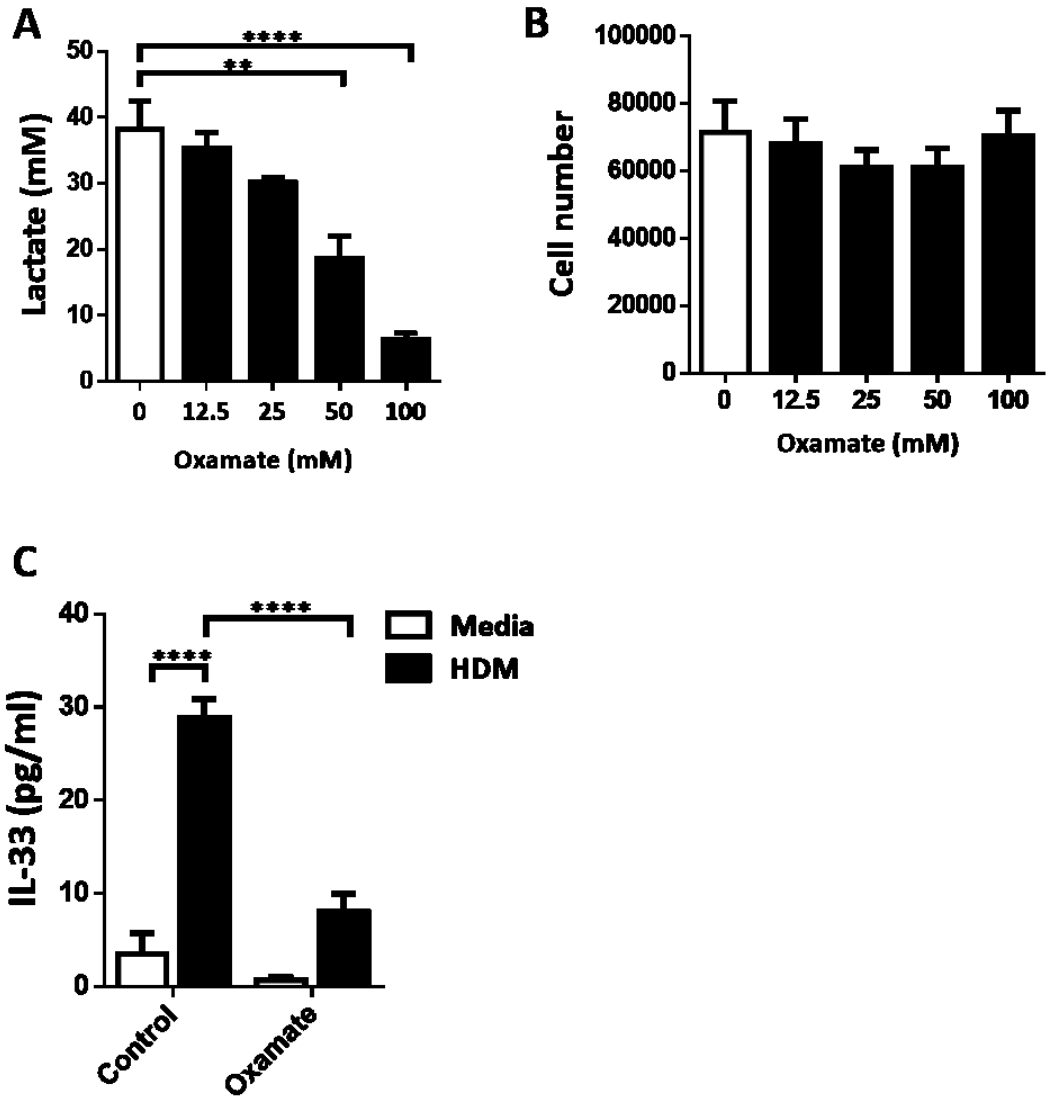


Figure 6: Inhibition of anaerobic glycolysis by oxamate diminished HDM-induced IL-33 secretion by airway epithelial cells. BEAS-2B cells were seeded in flat-bottom 96-well plates at a density of 20,000 cells/well and then serum starved for 24 hours. They were then treated with 12.5 mM, 25 mM, 50 mM and 100 mM oxamate for 1 hour for (A) supernatant lactate level measurement and (B) cell number determination (Trypan blue). According to these data, 50 mM was chosen as the effective dose of oxamate. BEAS-2B cells were then seeded at 20,000 cells/well, serum starved for 24 hours, pre-treated with media or 50 mM oxamate for 1 hour followed by treatment with either media control or HDM (100 μg/mL) for 2 hours. Cell culture supernatants were collected for (C) IL-33 measurement. Data presented as mean values ± SEM for biological replicates n=6, **p<0.01, ****p<0.0001 (one-way ANOVA and Dunn's post test).

Figure 6: Inhibition of anaerobic glycolysis by oxamate diminished HDM-induced IL-33 secretion by airway epithelial cells.



Chapter 4: Allergen driven rapid increases in glycolytic flux facilitates cytoskeleton dependent IL-33 translocation and release.

4.1 Introduction

In Chapter 3 we demonstrated an early rapid increase of glycolysis in airway epithelial cells in response to HDM that facilitates secretion of type-2 promoting cytokine IL-33. However, the signaling pathways regulating allergen-induced glycolysis and IL-33 release remain unclear. Although IL-33 was originally thought to be released during cellular damage/injury due to its localization inside the nucleus [248], several recent studies have revealed that exposure to endogenous extracellular danger signals or exogenous environmental stimuli could also lead to rapid live cell release of pre-formed IL-33 from both the nucleus and vesicular compartments in which this cytokine resides [244-247]. Researchers found that IL-33 was located both in the nucleus and membrane-bound cytoplasmic vesicles, and underwent dynamic nuclear-cytoplasmic flux in fibroblasts [247], indicating its intracellular inter-organelle translocation. However, IL-33 does not have a traditional secretion signal sequence [251]. The current data suggests that its release is likely through some unconventional mechanism depending on the nuclear pore complex [247] and intracellular calcium mobilization [244].

Unconventional release of cytokines that do not have a signaling peptide such as IL-1β [400] is not rare. Our understanding of the processes governing unconventional release of proteins is still limited, but an intact cytoskeletal network appears to be required [247, 401]. This is consistent with the general idea that the

cytoskeleton forms tracks for vesicle and organelle transport, as well as providing expelling or stabilizing forces for secretory compartments when needed [402-404]. Since intracellular calcium mobilization plays an important role in cytoskeleton rearrangement [405], taken together with the published data on IL-33 release, it is likely that IL-33 secretion is mediated by intracellular calcium regulated cytoskeletal rearrangement.

As we show that inhibition of cellular glycolysis with DCA and oxamate inhibits IL-33 release (Chapter 3), we argue based on their respective targets that either ATP and/or lactic acid produced as a result of glycolysis may contribute to HDM-induced IL-33 release. Further support for a role for ATP in IL-33 release is suggested by its known role in facilitating motor protein function and activities [406]. ATP is mainly generated in the cells from glucose via glycolysis and OXPHOS. Although OXPHOS is the central pathway in generating ATP in the presence of sufficient O_2, cells may preferentially use glycolysis for ATP generation even without oxygen deprivation based on certain needs [331]. One example is that glycolysis derived ATP fuels the demand of membrane associated actions (e.g., protein translocation) in a timely manner compared to OXPHOS derived ATP, as it is generated in the cytosol and is in closer proximity to membrane compartments [332]. As a result, we hypothesize that HDM-induced rapid increases in glycolysis may provide glycolytic ATP to facilitate the energy demands for the rapid translocation and secretion of IL-33 from epithelial cells. Specifically, the cells may undergo energy deprivation after HDM exposure due to the increased energy consumption required for IL-33 translocation from the nucleus to the cytoplasm and

extracellular transport. This energy deprivation would lead to the activation of AMPK, a well known regulator of cellular glucose metabolism that is able to promote pathways producing ATP [353] such as GLUT1-mediated glucose uptake [354] and glycolytic flux [407].

While we hypothesize that glycolysis provides the necessary energy for epithelial cell responses to HDM exposure, the contribution of glycolysis-generated lactate and extracellular acidity to IL-33 release is also worthy of further analyses. Although no direct evidence has linked IL-33 production with extracellular acidity or lactate, an earlier study has reported that extracellular acidosis could function as an endogenous danger signal to induce cytokine release by macrophages [408]. Considering that extracellular acidification occurs during glycolysis, it may play a role in glycolysis-associated IL-33 release. Lactate is also known as a signaling molecule recognized by GPR81 to regulate cell proliferation and survival [348]. Understanding whether such signaling is related to IL-33 secretion is needed.

Although our data suggest that HDM induces IL-33 through metabolic alterations in the epithelium, the HDM specific receptors or pathways initiating these events remain unknown. Airway epithelial cells recognize external stimuli through a wide array of PRRs. It is proposed that airway epithelial cells initiate allergic airway responses by recognizing specific allergenic components which drive the release of endogenous danger signals [409]. Despite the current understanding that epithelial cells express various TLRs that recognize viral antigens and allergenic proteins to induce the release of inflammatory cytokines such as tumor necrosis factors (TNF), IL-6 and interferons (IFN) [378], much remains unknown regarding

the receptor(s) that mediate HDM-induced IL-33 release. Previously, several studies have demonstrated a potential correlation between TLR signaling and *Il33* expression by showing that agonists to TLR3, TLR4 and TLR5 increased mRNA expression of *Il33* [410-412]. Moreover, the MyD88 independent TLR downstream signaling molecule TBK1 has been implicated in TLR4 agonist induced rapid induction of glycolysis and cytokine secretion [338]. However, such studies only focused on the regulation of *Il33* transcription, rather than live cell secretion. Recently, two groups of researchers have shown that IL-33 secretion from live cells was mediated by extracellular ATP released from epithelial cells in response to fungal allergen-derived proteases [244, 245]. These results suggest that P2 purinergic receptor signaling may be important in IL-33 release, and that protease properties of allergens may account for IL-33 secretion by airway epithelial cells [249, 389]. Although direct evidence for HDM-induced ATP release is lacking, the observations that ATP is elevated following *in vivo* exposure to HDM in mice [245] and that HDM is reported to contain protease activity [224] supports the hypothesis that these pathways/mechanisms might account for HDM induction of IL-33 and glycolytic flux.

Moreover, PRR mobilization of intracellular calcium might be involved in HDM-induced IL-33 and glycolytic flux as calcium signaling has been shown to play a critical role in epithelial cell release of IL-33 [244, 245]. In fact, formyl peptide receptor 2 (FPR2), a widely expressed G-protein coupled receptor [413], has been shown to function as a pattern recognition molecule through its ability to recognize various lipid and protein ligands [414-418]. It has also been shown to contribute to

allergic airway inflammation [416]. As FPR2 mediates Ca^{2+} mobilization, immune cell migration and mediator release [413], it might be a potential candidate for the mediation of HDM-induced IL-33 release and glycolytic flux.

To address these issues, we asked the following questions: (1) how does glycolysis contribute to the release of IL-33? (2) What are the pattern recognition receptors mediating HDM-induced IL-33 and glycolytic flux?

4.2 Results

4.2.1 HDM-induced rapid release of IL-33 was dependent on cytoskeleton activity and Rac1 signaling.

To test the proposed hypothesis that HDM-induced IL-33 release is dependent on cytoskeleton-mediated translocation, we first examined the pattern of IL-33 translocation induced by HDM. To capture the dynamic processes involved in HDM-induced IL-33 release, we treated BEAS-2B cells with HDM (100 μg/mL) for 15 minutes, collected the supernatants and isolated proteins from nucleus, cytosol and cytoskeleton attached compartments. We found that under unstimulated conditions, IL-33 resided in both the cell nucleus and cytosol (Fig. 7A). Proportionally speaking, more IL-33 resided in the nucleus (about 57%), with less residing in the cytosol (about 33%). HDM exposure resulted in a rapid export of IL-33 from both the nucleus and cytosol (Fig. 7B), consistent with the recently pulished data that IL-33 translocated from the nuclear to cytosolic compartments and then to the extracellular milieu [247]. Interestingly, when cells were pre-treated with 20 mM DCA, a significant increase in IL-33 was observed in the cytoskeletal compartment.

Thus, the significant reduction in IL-33 in the supernatant caused by DCA was due to the significant increase of IL-33 attached to the cytoskeleton (Fig. 7C).

To further explore the role of the cytoskeleton in IL-33 release, we pre-treated BEAS-2B cells with the inhibitor of Rac1, a small molecule Rho-GTPase that plays an essential role in actin rearrangement and exocytosis [419], followed by either media or HDM (100 μg/mL) treatment for 2 hours. Based on the initial study showing that the chemical compound NSC23766 inhibited Rac1 activity by more than 90% at a concentration of 100 μM [420], we treated cells with two different concentrations of the inhibitor at 50 μM and 0.5 μM, and 50 μM was expected to inhibit Rac1 activity by 50% [420]. We found that HDM-induced IL-33 release by BEAS-2B cells was significantly reduced following treatment of cells with 50 μM Rac1 inhibitor (Fig. 7D) independently of cell viability changes (Fig. 7E). Similarly, the increase in lactate secretion in HDM-exposed cells was reduced by Rac1 inhibition (Fig. 7F), indicating that cytoskeleton rearrangement may be upstream of the induction of glycolysis. To confirm the effects of the Rac1 inhibitor, we showed that siRNA knockdown of Rac1 (Fig. 7G) inhibited IL-33 release (Fig. 7H). Changes in intracellular calcium have been shown to induce the activation of Rac1 [419] and to mediate IL-33 release [244, 245]. Thus, we asked whether calcium chelation with EDTA [421] could alter allergen-driven IL-33 release. Based on the previously published data that 0.1 mM EDTA was sufficient to chelate extracellular calcium and 1.0 mM was required to inhibit intracellular calcium [422], we treated BEAS-2B cells with a mid range dose (0.5 mM) of EDTA for 30 minutes, followed by media or HDM

(100 μg/mL) treatment for 30 minutes. Consistent with the literature, calcium chelation reduced the level of IL-33 release (Fig. 7I).

4.2.2 HDM-induced rapid release of both full-length and cleaved IL-33 that was dependent on endoplasmic reticulum (ER)-Golgi trafficking.

In addition to the involvement of cytoskeleton activities, IL-33 secretion is also likely mediated by intracellular vesicle trafficking from the nucleus to the cytosol, and then to the extracellular milieu [247]. To confirm this, the effects of ER-Golgi vesicular trafficking inhibition with Exo1 [423] on IL-33 release was determined. BEAS-2B cells were treated with DMSO as a control or 20 μM Exo1 (the half minimal inhibitory concentration, or IC_{50}, of Exo1 [423]) for 30 minutes, followed by media or HDM (100 μg/mL) exposure for 2 hours. While HDM exposure significantly induced IL-33 release from airway epithelial cells, Exo1 treatment significantly reduced the IL-33 release (Fig. 8A), suggesting the participation of ER-Golgi trafficking in IL-33 release.

A recent study analyzing IL-33 live cell secretion induced by HDM exposure suggests that IL-33 is processed into the mature form by calpain-2 [245], a process considered to occur in the ER-Golgi complex [424]. The participation of ER-Golgi trafficking we observed indicates the potential requirement of proteolytic processing for IL-33 release. To confirm this hypothesis, we then concentrated the cell culture supernatants to obtain total secreted proteins and analyzed the secreted forms of IL-33 by western blot. Both full-length IL-33 and the cleaved isoform were seen on the gel. Interestingly, when cells were pre-treated with DCA, the amount of

secreted full-length protein of IL-33 was reduced (Fig. 8B – C). This data confirmed the observation that DCA treatment reduced HDM-induced IL-33 release by BEAS-2B cells, while its limited effects on the secretion of cleaved IL-33 suggest potentially different mechanisms for the release of full-length and cleaved IL-33.

4.2.3 HDM-induced increase of anaerobic glycolytic flux provided glycolytic ATP to facilitate cytoskeleton-mediated IL-33 release.

Since IL-33 release from airway epithelial cells was likely mediated by cytoskeleton-dependent translocation over a very short time frame, we hypothesized that ATP derived from glycolysis rather than, or in addition to, that generated through OXPHOS would be required to fuel cytoskeleton-mediated IL-33 release. To address the hypothesis that HDM-induced glycolysis provides glycolytic ATP for the release of IL-33, we first defined the roles of intracellular ATP from different sources on IL-33 release. Specifically, BEAS-2B cells were seeded at 20,000 cells/well and cultured overnight in media supplemented with a direct substrate of glycolysis – glucose, or the direct substrate of OXPHOS – pyruvate. 24 hours later, the cells were treated with media or HDM (100 μg/mL), and IL-33 levels in the supernatants were determined. HDM induced significant release of IL-33 in cells supplemented with glucose, whereas those bathed in pyruvate-supplemented media secreted significantly lower levels of IL-33 (Fig. 9A). To further manipulate the intracellular ATP from different sources, we pre-treated the cells overnight with dimethyloxaloylglycine (DMOG) (1mM), an inducer of anaerobic glycolysis by inhibiting the hypoxia-inducing factor (HIF)-hydroxylase [425], resulting in

increased glycolytic ATP production. These cells were then treated with HDM (100 μg/mL) for 2 hours for IL-33 measurement. We saw that increasing glycolytic ATP production by DMOG enhanced HDM-induced release of IL-33 (Fig. 9B). Direct inhibition of glycolytic ATP and OXPHOS ATP was performed respectively using the LDH inhibitor oxamate (50 mM) and the mitochondrial ATPase inhibitor oligomycin (4 μM) followed by 2 hours HDM (100 μg/mL) exposure. Our data demonstrated that only the inhibitor of glycolytic ATP production (oxamate) had any effect on the early release of IL-33 driven by HDM treatment (Fig. 9C – D).

To understand whether AMPK accounts for the rapid induction of glycolysis to fuel the need of cells for IL-33 release, pharmacological inhibition and siRNA knockdown of AMPK were carried out. We first utilized the potent AMPK inhibitor compound C (AMPKi). Compound C has been widely used in blocking AMPK at the concentration of 20 μM [426, 427]. Based on these data, blockade AMPK blockade with Compound C at various concentrations (10 μM and 0.1 μM). 10 μM compound C pre-treatment resulted in significant reduction of HDM-induced IL-33 release, while the reduction caused by 0.1 μM compound C was smaller, suggesting a concentration-response relationship between these 2 concentrations. Significant reduction of HDM-induced lactate production was also observed. (Fig. 9E & G), which was independent of cell death (Fig. 9F). However, compound C has also been reported to have other potential targets including bone morphogenetic protein (BMP) and mTOR signaling [428, 429]. To validate the effects of AMPK blockade on IL-33 release, we then conducted the siRNA knockdown of AMPK. The same reduction of HDM-induced IL-33 release was observed with siRNA (Fig. 9H – I).

4.2.4 Extracellular acidity or lactate did not play regulatory roles in HDM-induced IL-33 release.

The current study shows that the HDM-induced immediate increase of glycolysis facilitates the rapid release of IL-33 from airway epithelial cells. To further understand the contribution of other micro-environmental changes resulting from increased glycolysis (e.g., extracellular acidification and lactate) to IL-33 release, we tested the involvement of extracellular acidity n HDM-induced IL-33 release by manipulating the extracellular microenvironment with a series of lower pH values in the presence of HDM. We used acetic acid, a weak acid that slightly modulated extracellular pH without introducing other ions or damaging cells, to reduce extracellular pH and mimic the slight acidification resulted from increased glycolysis. The cells were serum starved, bathed in media with different pH values (pH at 7.5 of the media was adjusted to 6.7, 5.8, 5.4 and 4.6) for 30 minutes, and then treated with either media or HDM (100 μg/mL) for 2 hours for IL-33 measurement. As the extracellular pH slightly decreased from pH 7.5 to pH 5.8, IL-33 release was enhanced. However, such an increase in IL-33 release was also seen in cells under normal culture conditions, indicating a general increase of baseline release of IL-33 independently of the presence of environmental stimuli (Fig. 10A).

To determine whether extracellular lactate played a role in IL-33 release, BEAS-2B cells were treated with different concentrations of lactic acid (2.5 mM, 5 mM, 10 mM, 20 mM and 40 mM) in addition to HDM (100 μg/mL) for 2 hours. The magnitude of HDM-induced IL-33 release was unchanged (Fig. 10B).

4.2.5 HDM-induced rapid release of IL-33 and glycolytic flux was likely mediated by FPR2 signaling.

To investigate the PRRs involved in HDM-induced IL-33 and glycolysis, we first examined the effects of blocking TLR signaling, as TLR4, TLR3 and TLR5 have been shown to contribute to *Il33* expression [410-412]. As the downstream signaling of these TLRs can be either MyD88-dependent or MyD88-independent [430], we utilized a specific inhibitor of MyD88 (Pepinh-MYD), to block the MyD88-dependent signaling, and an inhibitor of TBK1 which is downstream of TLR activation, but not MyD88 [431], to block the MyD88-independent signaling. Specifically, BEAS-2B cells were seeded at 20,000 cells/well and treated with 10 μM Pepinh-MYD or its control peptide Pepinh-Control for 30 minutes. Pepinh-MYD contains a peptide sequence from the MyD88 TIR homodimerization domain and has been engineered to enable its translocation through the cell membrane to intracellularly block MyD88 [432]. Treatment of cells with Pepinh-MYD at 10 μM has been previously shown to effectively block MyD88 dependent TLR signaling by multiple studies [433, 434]. The cells were then treated with media or HDM (100 μg/mL), and IL-33 and lactate were measured in the supernatants after 2 hours of HDM treatment. We observed a consistent induction of both IL-33 and lactate by HDM stimulation as compared to the media controls. However, there was little reduction of HDM-induced IL-33 and lactate by MyD88 inhibition (Fig. 11A – B). We then treated BEAS-2B cells with either DMSO as a control or 5 μM TBK1 inhibitor, BX795, followed by 2 hours exposure to HDM (100 μg/mL). We saw concomitantly

induced IL-33 and lactate by HDM exposure, which were both significantly reduced in the presence of the TBK1 inhibitor (Fig. 11C – D). However, the reduction of IL-33 was not reproduced in TBK1 siRNA-treated cells, suggesting that the effects of the inhibitor were not specific (Fig. 11E – F).

Extracellular ATP and its receptor, the P2 purinergic receptor, have been shown to mediate epithelial cell secretion of IL-33 induced by *Alternaria* [244, 245], but the evidence for this pathway in HDM-induced IL-33 secretion is lacking. To address the role of extracellular ATP and its receptor in our model, we first measured ATP release from 16HBE cells after 2 hours exposure to HDM (100 μg/mL). We did not observe a significant induction of extracellular ATP following HDM exposure in these cells (Fig. 12A). To further examine this pathway, we analyzed the effect of blocking the P2Y2 receptor with the inhibitor suramin [435]. According to initial studies analyzing the effects of suramin in blocking extracellular ATP induced signaling, researchers found that suramin inhibits activities of P2 purinergic receptor at concentrations above 10 μM [436], and the blocking efficiency reaches 80% at 100 μM [437]. Based on these data, BEAS-2B cells were treated with 100 μM suramin, 50 μM ATP, or 100 μM suramin combined with 50 μM ATP for 30 minutes followed by 2 hours of exposure to media or HDM (100 μg/mL). We found that neither suramin nor ATP treatment had any effect on HDM-induced release of IL-33 (Fig. 12B).

Earlier studies identifying extracellular ATP as a regulator of IL-33 release claimed that the protease activity of the fungal allergen *Alternaria* was the cause of the ATP release [244, 245]. Thus we then sought to determine whether protease

activity played a role in HDM-induced IL-33 release. We heated HDM at 60˚C for 30 minutes to deactivate its protease activity and compared its induction of IL-33 to that of non-heated HDM. BEAS-2B cells were treated with both heated and non-heated HDM. IL-33 levels in the supernatants were determined after 2 hours exposure. Notably, we did not see any differences in the capability of HDM to induce IL-33 release after heating (Fig. 12C), suggesting that HDM likely does not induce extracellular ATP release through its proteolytic activity.

As we observed that IL-33 release was regulated by intracellular calcium mobilization, PRRs that induce the intracellular calcium flux might be potential candidates that mediate HDM-induced IL-33 release. As FPR2 was found to contribute to allergic airway inflammation [416], and to mediate Ca^{2+} mobilization, immune cell migration and mediator release [413], we hypothesize that FPR2 may regulate HDM-induced IL-33 release and glycolysis. To test this hypothesis, we utilized the FPR2 antagonist WRW4, which specifically inhibits the extracellular binding of FPR2 to its peptide agonist [438]. BEAS-2B cells were first treated with DMSO control or 20μM FPR2 antagonist WRW4 (based on the published effective concentration for more than 90% inhibition of FPR2 agonist binding [438]) for 30 minutes followed by either media control or HDM (100 μg/mL). Cell culture supernatants were collected at 30 minutes post-exposure for IL-33 measurement. Cells were then seeded at a density of 12,000 cells/well for live cell respiration measurement (Seahorse). They were pretreated with 20 μM WRW4 for 30 minutes. Glycolysis was then monitored as ECAR following the manufacturer's instructions, and HDM was injected before the 4^{th} measurement. Blockade of the FPR2 pathway

significantly reduced HDM-increased IL-33 release (Fig. 13A). Consistently, the HDM-induced enhancement in glycolytic flux was significantly reduced upon inhibition of FPR2 signaling pathways. These data suggest that FPR2 signaling pathways play an important role in HDM-mediated IL-33 release (Fig. 13B).

4.3 Discussion

4.3.1 Allergen induces cytoskeleton-mediated IL-33 translocation, which is also dependent on post-translational modification of the protein.

In the present study, we made the novel observation that HDM-induced rapid increases in glycolysis was at least partially responsible for the rapid, early release of IL-33 from epithelial cells. Moreover, our data demonstrated that IL-33 was rapidly translocated from its primary storage site in the nucleus to the cytoplasm following exposure to environmental allergens (Fig. 7B). Our findings that inhibition of glycolysis led to IL-33 trapping within the cytoskeleton confirms the role of the cytoskeleton in IL-33 release [247], and demonstrates for the first time that glycolysis may regulate this process in the context of IL-33 release.

Our studies provide a potential mechanism of how allergen regulates the movement of IL-33 from the cytoplasm to the extracellular milieu in that we demonstrated that both Rac1 and calcium were required for IL-33 release (Fig. 7D, E, G – H). Considering that these molecules are part of the same pathway in regulation of cytoskeleton activity, it is possible that certain signaling induce mobilization of intracellular calcium, resulting in increased Rac1 activation and then cytoskeleton facilitated cytokine secretion. Our data for the first time revealed that

IL-33 live cell secretion was dependent on Rac1 signaling. This appears to be contrary to published literature showing that Rac1 inhibition induced *Il33* expression [439]. However, previous studies did not examine the role of Rac1 in HDM-induced live cell secretion of IL-33, which may be due to very different regulatory mechanisms than *Il33* transcription. More importantly, the fact that HDM exposure did not induce *Il33* mRNA expression in airway epithelial cells in published studies [245, 439] supports our argument that HDM might induce Rac1 activation and then release of pre-formed IL-33 in the cells. Taken together, we suggest that HDM induces increases in intracellular calcium levels, which activate Rac1, leading to rapid cytoskeleton rearrangement and the translocation of IL-33 out of the cells.

In addition to proving that the cytoskeleton is required for HDM-induced IL-33 release, we further showed that inhibition of ER-Golgi trafficking blocked IL-33 secretion (Fig. 8A). This is surprising since it was proposed that cytokines lacking a conventional signaling peptide, such as IL-33, may utilize secretory pathways independently of ER-Golgi-mediated trafficking [440]. However, ER-Golgi trafficking allows protein quality control, surface molecule export as well as post-translational modifications. Multiple lines of evidence have suggested that IL-33 undergoes post-translational modification prior to secretion [243, 252]. Specifically, IL-33 requires proteolytic processing by calpain for maturation [243], which has recently been found to be involved in HDM-induced IL-33 release [245]. *Ex vivo* analysis revealed more possible isoforms that could be generated from full-length IL-33 [89] in addition to mature and immature forms. As a result, cleavage of IL-33 mediated by ER-Golgi trafficking might be required for HDM-induced IL-33 release of certain

forms of the protein. The fact that blockade of ER-Golgi trafficking did not fully inhibit IL-33 release would support this contention. Our data showing that both full-length and cleaved forms of IL-33 were present in the cell culture supernatants after HDM treatment further suggested that multiple forms of IL-33 were generated during HDM-induced release.

However, the biological activity of the cleaved IL-33 in our model remains unclear, as different processing mechanisms, which generate either inactivated or activated forms of IL-33 have been reported [243, 252]. Our current data is not sufficient to draw a conclusion on the biological activity of the cleaved IL-33. The fact that DCA treatment reduced the levels of only the full-length protein of IL-33 in the supernatant (Fig. 8B – C) was unexpected. However, it is reasonable to assume that glycolysis affects only part of the total cellular release of IL-33, and the cleaved shorter protein is released via a different mechanism. This observation is inconsistent with a recent publication reporting that HDM induces only the release of the cleaved isoform of IL-33 (18kD) from NHBE cells [245]. The discrepancy between our data and the published data might be explained by the differences in the cell culture systems used, as we utilized BEAS-2B, not NHBE cells in our studies. To address these differences and elucidate the pathways involved in intracellular processing of IL-33, future studies analyzing IL-33 release by primary human and animal cells, as well as in the BAL of mice are needed. However, even though full-length IL-33 has been reported to play different roles than mature IL-33 *in vivo* [441], this data does not reduce the significance of our findings. It was reported that the secreted full-length IL-33 could undergo proteolytic processing by proteases

derived from pro-inflammatory cells during the progression of allergic inflammation, generating isoforms with 10-fold greater potency to activate ST2 than full-length IL-33 [89]. As a result, inhibition of both full-length and mature cleaved IL-33 should confer protective effects against type-2 immune responses *in vivo*.

4.3.2 The energetic requirement for IL-33 translocation induces rapid glycolytic flux.

Our data suggested that the induction of glycolytic ATP production by HDM exposure was likely required to meet the energetic requirements for cytoskeletal rearrangement and IL-33 release from the epithelium. This hypothesis was supported by the finding that blocking Rac1 signaling inhibited lactate production (Fig. 7F), suggesting that HDM-induced glycolytic flux was probably downstream of Rac1 activated cytoskeleton rearrangement. As reviewed earlier, cytoskeletal rearrangement consumes ATP and may lead to an immediate and transient ATP deprivation in the cells. This immediate energy deprivation after HDM exposure might cause induction of glycolytic flux, which in turn provides glycolytic ATP and satisfies the bioenergetic needs to facilitate IL-33 release. Our data did show that enhancement of glycolytic ATP caused an increase in HDM-induced IL-33, and *vice versa*. At the same time, manipulation of OXPHOS ATP did not alter the magnitude of HDM-induced IL-33 secretion (Fig. 9A – D), supporting the contention that airway epithelial cells required glycolytic ATP for its secretion of IL-33. These data for the first time show that the allergen-induced rapid IL-33 release preferentially depends on glycolytic ATP as the energy source. This finding is consistent with the long held

belief, but important concept that glycolysis-derived ATP plays different physiological roles than ATP generated through OXPHOS [332], and the initial phase of cellular secretion depends on glycolytic ATP [442].

Since the increase of glycolysis is thought to arise as a result of a rapid increased energy demand, AMPK, the molecule sensing shortages of ATP [354, 355, 407], is likely the inducer of glycolysis in this case. Our data with AMPK inhibition using both a pharmacological inhibitor and genetic modification supported this hypothesis (Fig. 9E – I). Although details on the phosphorylation status of AMPK remain to be identified, the involvement of AMPK in this pathway is strongly indicated. Currently, AMPK is mainly implicated in metabolic disorders such as obesity and diabetes [443]. Our data for the first time suggests that it may also play a role in IL-33 release in the lung, which greatly advances our knowledge of the interplay between metabolism and immune function. Although our study has provided strong support for a link between glycolysis and IL-33 release, several aspects remain to be elucidated. First, the causal links between the identified pathways remain to be established. Although IL-33 translocation regulated by Rac1-mediated cytoskeleton activity is strongly suggested, staining of intracellular IL-33 in the presence of a Rac1 inhibitor is needed to obtain direct evidence for this hypothesis. Also, the mechanism that AMPK induces glycolysis needs to be confirmed by (1) demonstrating that the AMP to ATP ratio is transiently increased by HDM exposure; and (2) confirming that AMPK phosphorylation leads to enhanced glycolytic flux. Moreover, since we propose that glycolysis induction was downstream of calcium signaling-mediated IL-33 release, it is important to analyze

whether the AMPK-mediated glycolysis also facilitates intracellular calcium dependent release of IL-33 by *Alternaria* [244, 245]. Although we did not observe an increase in lactate levels in the cell culture supernatants after *Alternaria* exposure (Fig. 4F), we cannot rule out the possibility that glycolytic flux was also involved in *Alternaria*-induced IL-33 release. For example, lactate might be abrogated by other *Alternaria*-induced responses (e.g., induction of mechanisms taking up extracellular lactate, the protease property interferes with LDH activities, etc.) in BEAS-2B cells, rendering lactate undetectable at the time we measured it in the supernatants.

Besides analyzing the above-hypothesized pathway, we also sought to understand whether changes in the cellular microenvironment that occurred secondarily to glycolysis played a role in IL-33 secretion, especially since both DCA and oxamate treatment inhibited lactic acid, extracellular acidification and glycolytic ATP, resulting in reduced IL-33 release. However, extracellular acidity seemed to enhance the baseline release of IL-33 rather than promoting HDM induction of IL-33 (Fig. 9A). This was not unexpected as extracellular acidity has only been reported to regulate IL-1β release induced by LPS from macrophages [408], in which the regulatory mechanisms are likely very different from that in airway epithelial cells. However, it is indeed interesting that increased extracellular acidity within the pH ranges from 5.8 – 7.5 caused an overall increase of IL-33 release from cells in both media control and HDM. We infer that an extracellular pH lower than 5.8 induces cellular stress, interfering in regular processes of host responses or even causes apoptosis that deactivates IL-33 [241]. In the meantime, the general increase of IL-33 production at moderate acidity suggests involvement of acidic organelles in IL-33

release. It is known that the release of some cytokines depends on secretory lysosomes [440], the dual functional organelle conferring degradation and secretion in non-professional secretory cells [444]. Considering that both our data and the literature suggest that IL-33 may be secreted via vesicle-mediated pathways [247], the general increase in IL-33 release by extracellular acidity may imply involvement of secretory lysosomes.

Our findings that pre-treatment of BEAS-2B cells with different concentrations of lactic acid did not modulate the magnitude of IL-33 induction by subsequent HDM exposure ruled out a role for extracellular lactate in HDM-induced IL-33 release. There have been no reports on the role of lactate signaling in the mediation of cytokine release. However, lactate has been shown to promote proliferation of CD4+ T cells [370], which is consistent with its roles in promoting tumor cell survival and proliferation [445], probably by acting as an alternative metabolism substrate when glucose accessibility is restricted [446]. In our model, it probably should be treated more as a readout of the metabolic state of the cell, rather than a direct regulator of epithelial cell function. However, we cannot rule out the possibility that it may function as another mediator secreted by airway epithelial cells that might contribute to allergic inflammation *in vivo* by promoting the proliferation of other immune and structural cells.

4.3.3 FPR2 signaling likely plays important roles in recognition of HDM allergens and subsequent IL-33 release.

Although our study provided a potential mechanism by which HDM might induce IL-33 release through the regulation of glycolysis, the upstream regulation of HDM-induced IL-33 release, e.g., surface receptor on airway epithelial cells that recognized HDM to mediate the induction of IL-33 and glycolytic flux, remained to be identified. Reports in the literature support the involvement of TLR3, TLR4 and TLR5 in exogenous antigen induced *Il33* mRNA expression [411, 412], indicating that a common pathway downstream of these receptors may play a role in epithelial recognition of foreign antigens that drive IL-33 induction. However, we found that neither inhibition of MyD88, the common downstream adaptor molecule of these receptors [447], nor TBK1, the common MyD88-independent signaling molecule downstream of TLR3 and TLR4, had significant effect on HDM-induced IL-33 and glycolysis (Fig. 11). These data indicated that TLR signaling was not the major PPR pathway involved in HDM-induced IL-33 release. This is not contrary to the literature, as there is no direct evidence showing that the activation of TLRs directly induces secretion of IL-33. Even though TLR4 signaling on pulmonary structural cells was shown to be required for HDM-induced allergic asthma in mice [31], no evidence has directly proven that epithelium TLR4 responses were important for IL-33 secretion or allergic asthma pathogenesis. In fact, our data is consistent with a recent publication showing that *Alternaria* induced BAL levels of IL-33 in mice were not affected by genetic depletion of MyD88 [244].

The inconsistency in our data between TBK1 inhibition with BX795 and siRNA was probably due to off-target effects of BX795, as it might also inhibit PDPK1 and Akt phosphorylation, the molecules likely involved in the regulation of cell

metabolism [448]. Overall, our finding of no effects of TBK1 on IL-33 release is not inconsistent with the literature, as no one has examined the role of TBK1 in IL-33 release or allergic asthma pathogenesis despite some evidence that it is required for *Il33* mRNA expression in murine embryonic fibroblasts [412]. TBK1 was reported to regulate LPS-induced rapid induction of glycolysis that mediated IL-6 production in DCs [338]. However, they did not specify whether the secreted IL-6 was attributed to newly synthesized or pre-formed proteins. Also, considering that LPS did not induce glycolytic flux in BEAS-2B cells (Fig. 4F), it is reasonable to conclude that the responding mechanisms to exogenous stimuli differ between airway epithelial cells and DCs. It is important to know that although TLRs play an important role in environmental stimuli-induced expression of *Il33*, rapid live cell secretion of IL-33 is likely mediated by different mechanism(s). As a result, the signaling pathways identified to date to play a role in the regulation of *Il33* mRNA expression cannot be simply generalized to the rapid secretion of this protein.

According to two recent published studies, environmental allergen-induced ATP release and autocrine activation of its P2 purinergic receptor account for the induction of IL-33 in airway epithelial cells [244, 245]. According to this hypothesis, excessive ATP is expected to enhance IL-33 release from the cells, which would then be reduced by the ATP receptor inhibitor suramin. However, we observed no induction of extracellular ATP secretion by HDM (Fig. 12A), and no difference in IL-33 release was seen when extracellular levels of ATP were modulated in the presence of HDM (Fig. 12B). The reason for the discrepancy between our data and these other studies is not known. However, the ATP release was measured in 16HBE

cells, an epithelial cell line that is potentially different from BEAS-2B cells and NHBEs that were used in the published studies. A positive control that induces ATP release from airway epithelial cells such as *Alternaria* should be included. Also, our studies lack the validation that suramin and/or extracellular ATP are playing the desired roles, and we do not have evidence for the expression pattern of P2 purinergic receptor on BEAS-2B cells compared to NHBEs used in the published studies. Nevertheless, there are no previous reports of HDM-induction of ATP release in the airway epithelium. The fact that most of their observations were made in an *Alternaria* model actually supports the notion that different allergens might mediate IL-33 release through different mechanisms [245]. Most importantly, in both our data and the published studies, extracellular ATP was observed at the nanomolar level [245]. The recent studies showing a link to extracellular ATP utilized much higher concentrations of ATP at micromolar levels, raising the question of physiological relevance. However, caution should be taken in interpreting our data as thorough analyses of extracellular ATP at different time points post-exposure are lacking, and a concentration-response relationship between HDM exposure and extracellular ATP levels should also be analyzed.

Consistent with there being allergen-specific pathways for IL-33 release, there is strong evidence that *Alternaria*, the fungal-derived allergen, induces IL-33 release through a serine protease dependent pathway [249]. However, when HDM was heated to inactivate the protease activity of its contents, the induction of IL-33 was not affected (Fig. 12C), indicating that HDM-induced IL-33 release was not dependent on protease activity as was seen with *Alternaria*.

Although the involvement of TLR signaling as well as endogenous ATP and its receptor is inconclusive, the involvement of FPR2 in HDM-induced IL-33 and glycolytic flux might be worthy of further investigation. We made the novel observation that the FPR2 pathway played a role in both IL-33 release and glycolytic flux (Fig. 13). This is consistent with the literature that FPR2 mediates Ca^{2+} mobilization, immune cell migration and mediator release [413]. Considering that IL-33 release is also dependent on intracellular calcium mobilization, our studies support the contention that FPR2 recognizes certain components in HDM, causing increased intracellular Ca^{2+} that is known to regulate IL-33 release by airway epithelial cells [244, 245]. Moreover, we demonstrate for the first time that FPR2 signaling regulates HDM-induced glycolysis (ECAR), suggesting its potential role in regulation of cellular glucose metabolism. However, the causal link between FPR2, calcium signaling, IL-33 translocation and glycolysis remains to be further established. Direct analysis showing that specific activation of FPR2 leads to increased intracellular calcium and IL-33 release is needed. Also, the ligands in HDM that are recognized by FPR2 are not known at this time. Since FPR2 is capable of binding to both lipid and peptide/protein ligands [449, 450] such as the lipid mediator lipoxin A_4 (LXA4) and the Th2-promoting protein SAA [451-454], a comprehensive study of HDM-induced IL-33 release via FPR2 signaling (e.g., the potential ligands of FPR2 in HDM) needs to be established.

4.3.4 General limitations of the current studies.

Despite the existing caveats in the current studies that have been discussed above, there are limitations of our data that require further validation and research.

First of all, the targets of the inhibitors/siRNA used should be validated with specific readouts. For all the inhibitors used in our analyses, the concentrations were selected based on published manuscripts, and were shown to specifically and effectively block the desired targets. However, we do not have direct data supporting our contention that their effects were due to the expected specific actions. To do this, Rac1-GTP activities and levels should be tested respectively in cells treated with the Rac1 inhibitor and siRNA, together with direct visualization of cytoskeleton rearrangement alterations in the presence of the Rac1 inhibitor, HDM and DCA. EDTA chelation of calcium should also be confirmed with intracellular calcium measurement. For the blockade of ER-Golgi trafficking by Exo1, visualizing IL-33 translocation will provide direct evidence for its effects. To confirm the successful blockade of AMPK signaling, the phosphorylation status at threonine 172 (Thr172) is the target readout for compound C, while the protein level should be confirmed following siRNA knockdown.

Similarly, the effects of inhibitors and/or siRNA against the PRRs and danger molecules should be validated. TBK1 phosphorylation status (serine 172, Ser172) and protein levels remain to be tested for validation of cellular response to TBK1 pharmacologial inhibition and siRNA knockdown. Meanwhile, as extracellular ATP and FPR2 signaling likely induce increased intracellular calcium flux, intracellular calcium recordings would be a reliable readout to prove specific blockade of FPR2, P2 purinergic receptors, and the action of extracellular ATP. In addition, a positive

control that induces ATP release should be included for further analyses. The expression pattern of P2 purinergic receptors on BEAS-2B cells should also be analyzed, as there is a possibility that these cells do not respond well to suramin or that extracellular ATP levels are low.

Another limitation of the current data is the lack of reproduction of our results in asthmatic and normal human epithelial cells. As discussed in Chapter 3, the current culture system has reduced generalizability for translating the observations into human airways. Our data suggested that primary normal human airway epithelial cells also exhibit increased glycolytic flux upon exposure to HDM. Based on this, we expect that similar mechanisms also mediate HDM induced IL-33 release in primary cells. Moreover, whether the same pathways account for the epithelium response to HDM in an air-liquid interface culture system should also be analyzed.

In summary, the current data, despite the discussed caveats and limitations, strongly support a scenario in which HDM induces the early release of IL-33 by first activation of FPR2, which then activates intracellular calcium mobilization and Rac1-mediated rapid cytoskeleton rearrangement. In turn, this process initiates translocation and release of IL-33 from both the nuclei as well as cytosolic compartments, and rapidly consumes ATP present in the cytosol, leading to an altered AMP/ATP ratio and activation of AMPK signaling. The AMPK signaling leads to increased glycolysis, which in turn generates glycolytic ATP to facilitate the early release of IL-33 (Fig. 14). Further *in vivo* analyses of cell metabolic-facilitated IL-33

release and its role in allergic asthma pathogenesis will reveal the role of cell metabolism in the pathogenesis of disease.

4.4 Figures

Figure 7: HDM-induced rapid release of IL-33 was dependent on cytoskeleton activity and Rac1 signaling. BEAS-2B cells were seeded in flat-bottom 6-well plates at a density of 100,000 cells/well and then serum starved for 24 hours followed by first treatment with DCA (20 mM) for 1 hour and then treatment with either media control or HDM (100 μg/mL) for 15 minutes. (A) – (C) IL-33 levels in the supernatant, nuclear, cytosolic and cytoskeleton-attached proteins from BEAS-2B cells were identified by ELISA. To determine if Rac1 was involved in HDM-induced IL-33 release, BEAS-2B cells were seeded in flat-bottom 96-well plates at a density of 20,000 cells/well, serum starved for 24 hours and then treated with either DMSO control or Rac1 inhibitor NSC23766 (Rac1i, doses shown in the graph) for 30 minutes followed by HDM (100 μg/mL). (D) IL-33 release, (E) cell viability and (F) lactate production were identified in the supernatants. BEAS-2B cells were transfected with Rac1 siRNA as described (Chapter 2, 2.15) and then treated with HDM (100 μg/mL) for 2 hours. (G) *Rac1* gene expression and (H) IL-33 induced by HDM exposure were determined. These cells were then treated with calcium chelator EDTA (0.5 mM) for 30 minutes followed by media or HDM (100 μg/mL) for 2 hours. (I) IL-33 levels in the supernatants were measured. Data presented as one representation of 3 experiments for (A) – (C), or as mean values ± SEM for biological replicates n=3-6, *$p<0.05$, ***$p<0.001$ (student's t test, one-way ANOVA and Dunn's post test).

Figure 7: HDM-induced rapid release of IL-33 was dependent on cytoskeleton activity and Rac1 signaling.

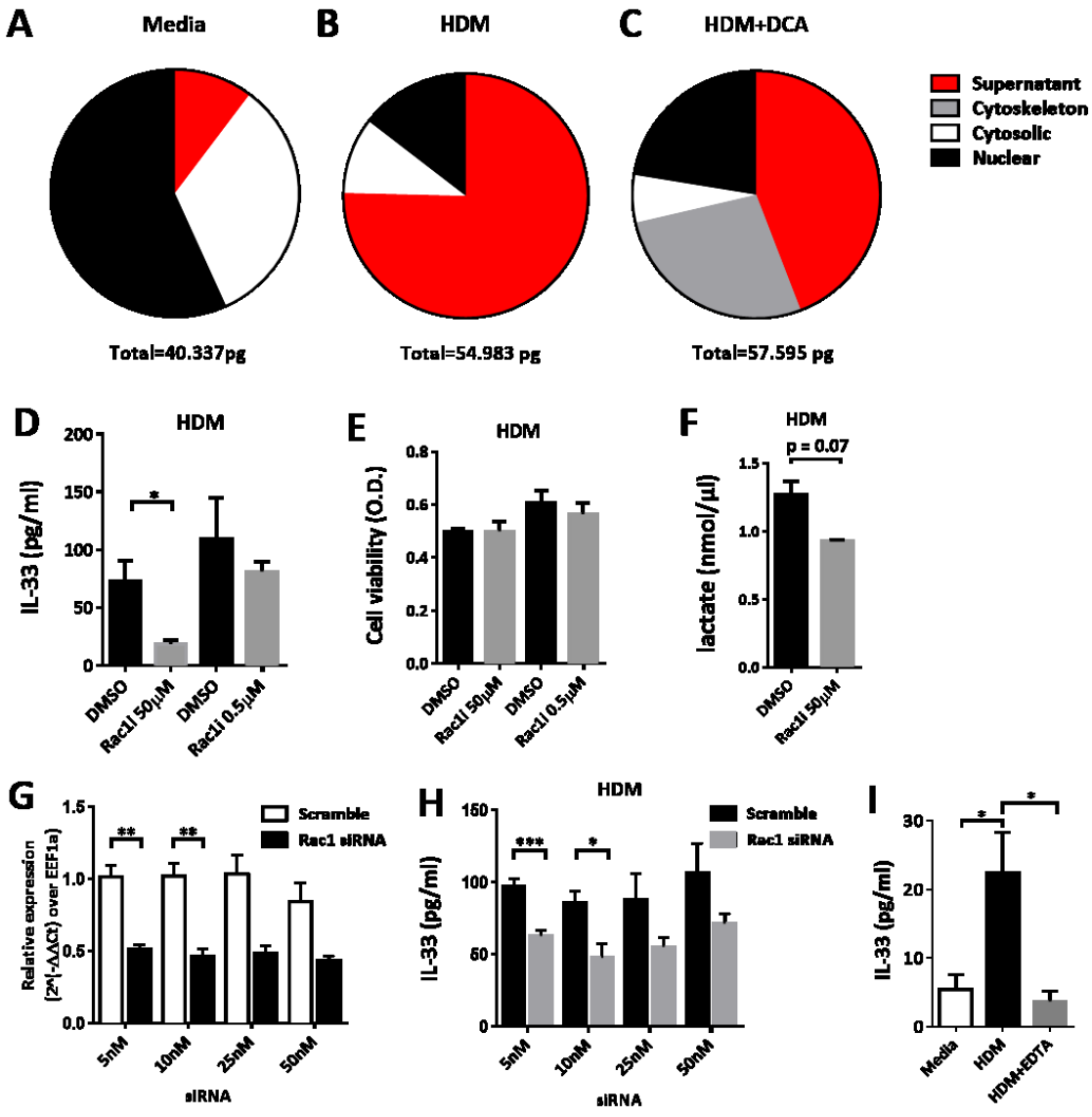


Figure 8: HDM induced rapid release of both full-length and cleaved IL-33 that was dependent on Endoplasmic reticulum (ER)-Golgi trafficking. BEAS-2B cells were seeded in flat-bottom 96-well plates at a density of 20,000 cells/well. They were then grouped to receive (A) either DMSO control or 20 μM Exo1 for 30 minutes. These cells were then treated with media control or HDM (100 μg/mL) for 2 hours. Cell culture supernatants were collected for IL-33 measurements. To identify whether the IL-33 proteins secreted were full-length or truncated, BEAS-2B cells were seeded in flat-bottom 6-well plates at the density of 100,000 cells/well. They were then treated with either media or DCA (20 mM) followed with either media control or HDM (100 μg/mL) for 2 hours. Cell culture supernatants were collected and concentrated by 100 folds using Amicon Ultra-0.5 mL Centrifugal Filters (EMD Millipore). (B) IL-33 in the concentrated supernatants was determined by Western Blot using anti-human IL-33 antibody (R&D), and (C) densities of the bands were quantified. Data presented as mean values ± SEM for biological replicates n=3-6, *p<0.05, **p<0.01, ****p<0.0001 (student's t test, one-way ANOVA and Dunn's post test).

Figure 8: HDM induced rapid release of both full-length and cleaved IL-33 that was dependent on Endoplasmic reticulum (ER)-Golgi trafficking.

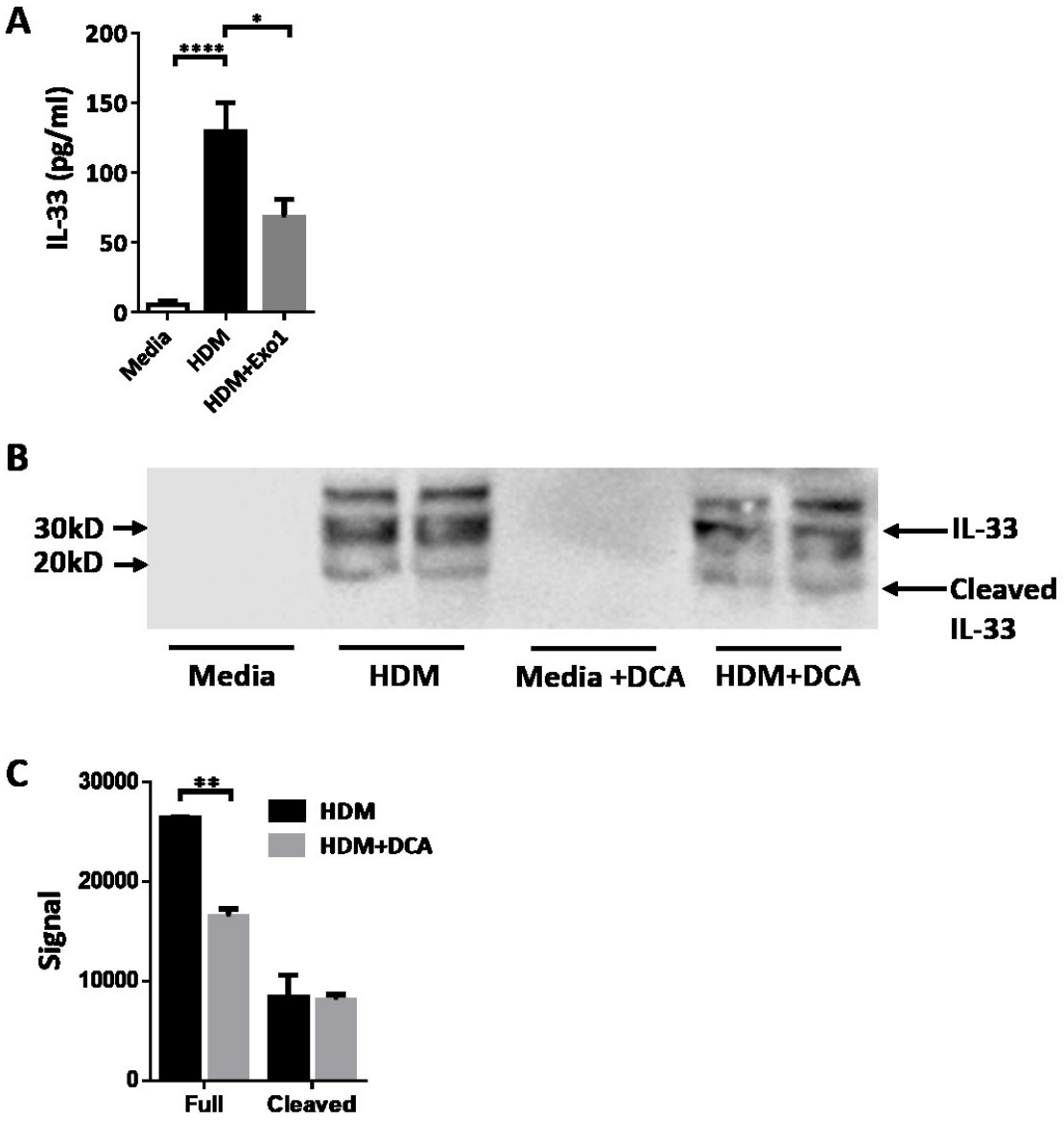


Figure 9: HDM-induced increase of anaerobic glycolytic flux provided glycolytic ATP to facilitate cytoskeleton-mediated IL-33 release. BEAS-2B cells were seeded in flat-bottom 96-well plates at the density of 20,000 cells/well. To test differential effects of glycolytic and OXPHOS ATP on IL-33 release, cells are cultured overnight in (A) pyruvate-supplemented, glucose-free media to reduce glycolysis or in (B) 1 mM DMOG to enhance glycolytic activities. These cells were then treated with HDM (100 μg/mL) for 2 hours and IL-33 levels were measured in the supernatants. To directly inhibit glycolytic and OXPHOS ATP production, cells were treated with (C) 20 mM sodium oxamate to inhibit LDH or (D) 4 μM oligomycin to inhibit mitochondrial ATPase followed by HDM for IL-33 release measurement. To determine if AMPK is involved in HDM-induced IL-33 release, cells are treated with either DMSO control or AMPK inhibitor compound C (AMPKi, doses shown in the graph) for 30 minutes followed by HDM (100 μg/mL). (E) IL-33 release, (F) cell viability and (G) lactate production were identified. AMPK siRNA knockdown was conducted and resulted (H) expression of *Prkaa1*, the gene encoding catalytic subunit of AMPK, as well as (I) IL-33 induced by HDM exposure were determined. Data presented as mean values ± SEM for biological replicates n=3-6, *p<0.05, ***p<0.001, ****p<0.0001 (student's t test, one-way ANOVA and Dunn's post test).

Figure 9: HDM-induced increase of anaerobic glycolytic flux provided glycolytic ATP to facilitate cytoskeleton-mediated IL-33 release.

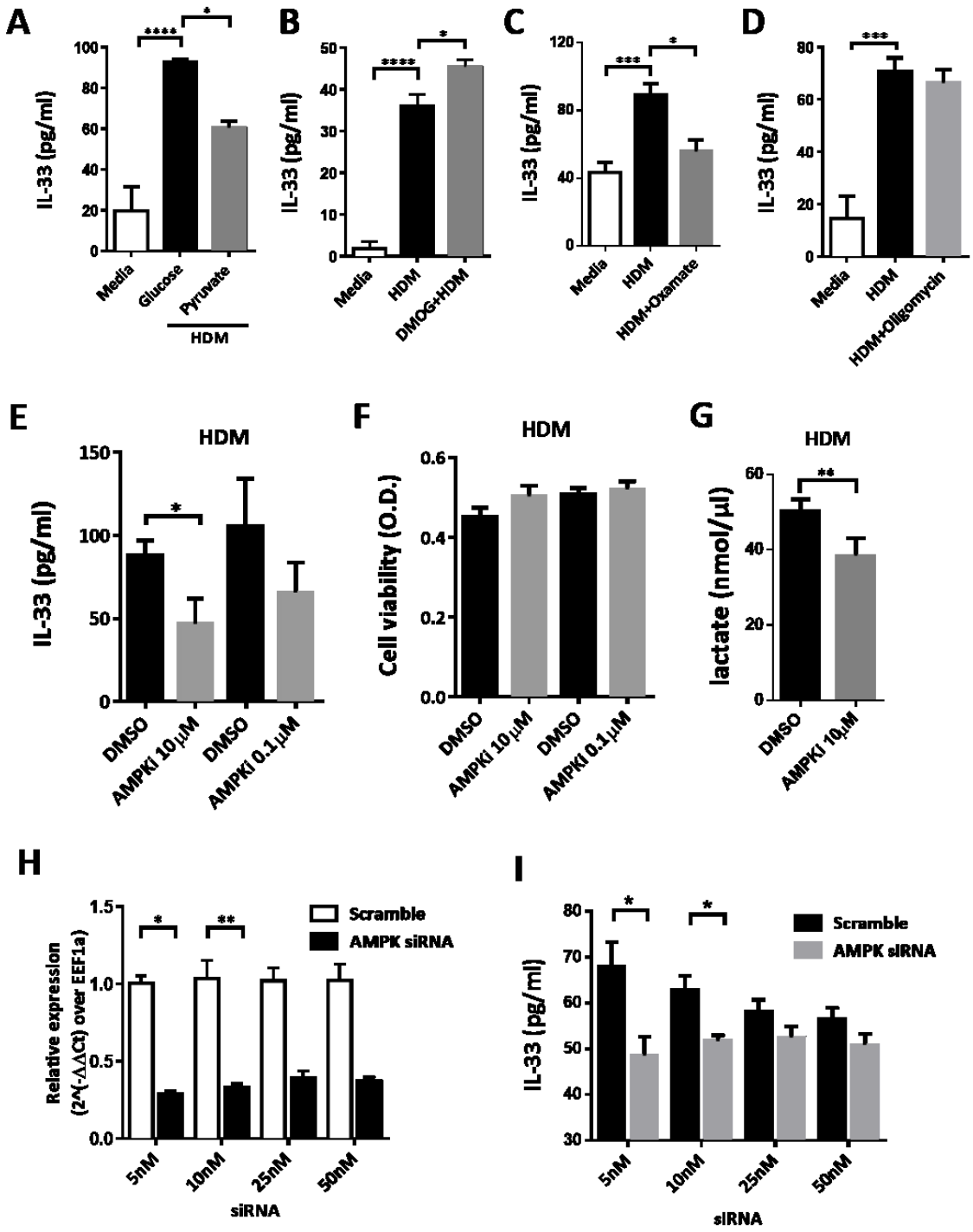


Figure 10: Extracellular acidity or lactate did not play regulatory roles in HDM-induced IL-33 release. To understand whether extracellular acidity contributes to HDM-induced IL-33 release, BEAS-2B cells were seeded at a density of 20,000 cells/well and serum starved. For modulation of extracellular acidity, acetic acid was added into cell culture media. Using the pH meter, the cell culture media pH (7.5) was adjusted to 6.7, 5.8, 5.4, and 4.6. Cells cultured in media with different pH were then exposed to HDM (100 μg/mL) for 2 hours. Cell culture supernatants were collected and (A) IL-33 was measured by ELISA. To test the effects of extracellular lactate, BEAS-2B cells were pre-treated with different doses of lactic acid (2.5 mM, 5 mM, 10 mM, 20 mM and 40 mM) for 1 hour, after which they were exposed to HDM (100 μg/mL). (B) IL-33 levels were determined in the supernatants 2 hours later. Data presented as mean values ± SEM for biological replicates n=6. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (student's t test, one-way ANOVA and Dunn's post test).

Figure 10: Extracellular acidity or lactate did not play regulatory roles in HDM-induced IL-33 release.

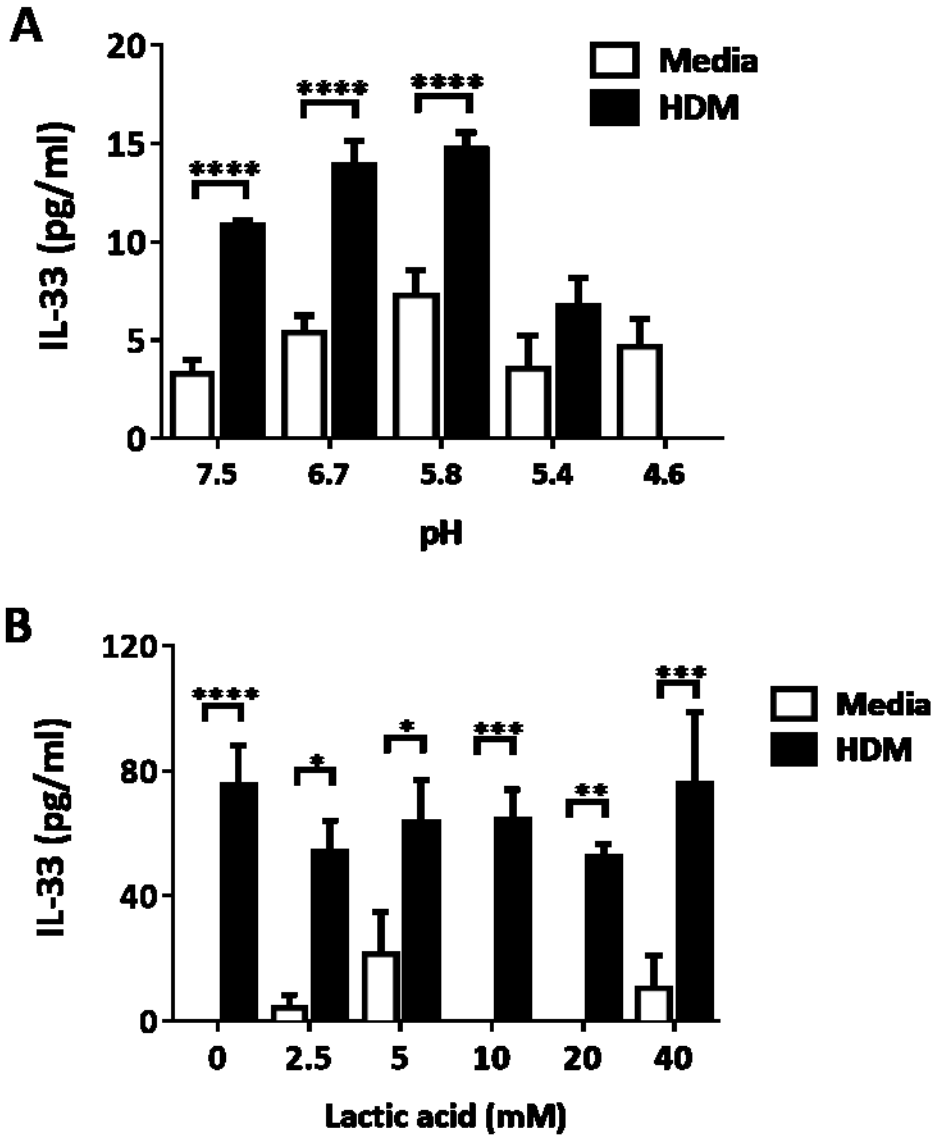


Figure 11: HDM-induced rapid release of IL-33 and glycolytic flux was independent of MyD88 and TBK1-mediated TLR signaling. BEAS-2B cells were seeded at a density of 20,000 cells/well and serum starved overnight. For the MyD88 inhibition, cells were treated with 10 μM Pepinh-MYD or its control peptide Pepinh-Control for 30 minutes followed by treatment with either media or HDM (100 μg/mL) for 2 hours. IL-33 (A) and lactate (B) were determined in the supernatants. For TBK1 inhibition, these cells were pre-treated with either DMSO control or 5 μM TBK1 inhibitor BX795 followed by 2 hours exposure to media or HDM (100 μg/mL) for IL-33 (C) and lactate (D) measurement. To validate the inhibitor results, cells were transfected with TBK1 siRNA for 48 hours, serum starved and then followed by 2 hours treatment by media or HDM (100 μg/mL). *Tbk1* expression was assessed by RT-PCR (E), and IL-33 in the supernatants was determined (F). Data presented as mean values ± SEM for biological replicates n=3-6, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (student's t test, one-way ANOVA and Dunn's post test).

Figure 11: HDM-induced rapid release of IL-33 and glycolytic flux was independent of MyD88- and TBK1-mediated TLR signaling.

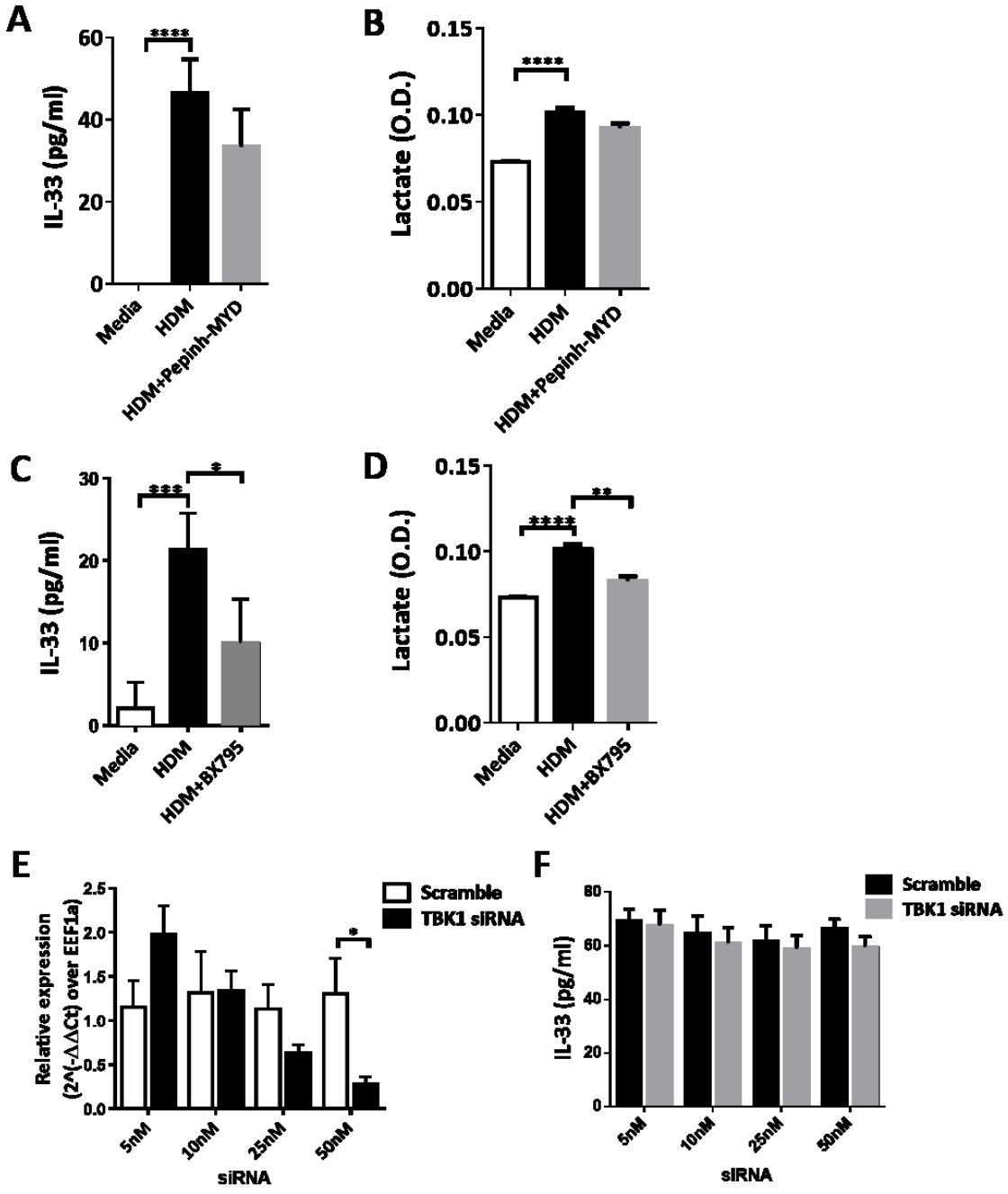


Figure 12: HDM-induced rapid release of IL-33 and glycolytic flux was not mediated by extracellular ATP. 16HBE cells were exposed to media or HDM (100 μg/mL) for 2 hours and ATP levels in the supernatants were determined (A). To understand whether extracellular ATP contributed to HDM-induced IL-33 release, BEAS-2B cells were treated with 100 μM suramin, 50 μM ATP, or 100 μM combined with 50 μM ATP for 30 minutes followed by 2 hours exposure to HDM (100 μg/mL). IL-33 in the supernatants was determined with ELISA (B). To test whether protease activity was involved, HDM was first heated to 60°C for 30 minutes to inactivate the enzymatic activities of its protease content. BEAS-2B cells were then exposed to regular and heated HDM (100 μg/mL) for IL-33 release measurement (C). Data presented as mean values ± SEM for biological replicates n=3-6, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (student's t test, one-way ANOVA and Dunn's post test).

Figure 12: HDM-induced rapid release of IL-33 and glycolytic flux was not mediated by extracellular ATP.

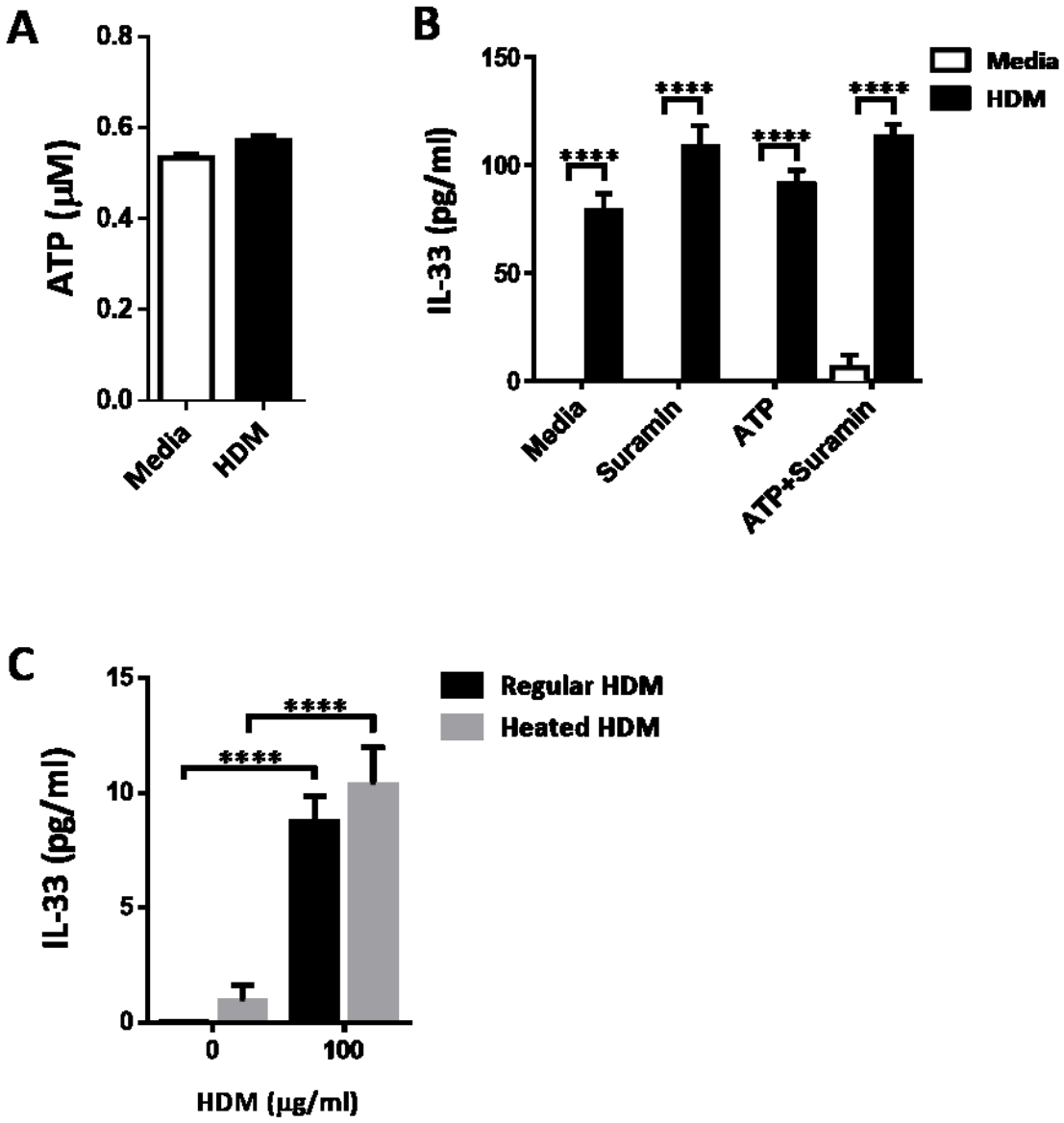


Figure 13: HDM-induced rapid release of IL-33 and glycolytic flux was likely mediated by FPR2 signaling. BEAS-2B cells were seeded in flat-bottom 96-well plates at a density of 20,000 cells/well and serum starved. They were then treated with DMSO control or 20 μM WRW4 for 30 minutes followed by either media control or HDM (100 μg/mL). Cell culture supernatants were collected at 30 minutes post-exposure for IL-33 measurement (A). Cells were then seeded at the density of 12,000 cells/well for live cell respiration measurement (Seahorse). They were incubated in a CO_2 free tank with buffer free media containing 20 μM WRW4 for 30 minutes and then loaded into Seahorse. ECAR was then measured following manufacture manual (Chapter 2.12) for 10 times within 70 minutes, and HDM was injected before the 4^{th} measurement (B). Data presented as mean values ± SEM for biological replicates n=3-6, *p<0.05, ***p<0.001, ****p<0.0001 (one-way ANOVA and Dunn's post test).

Figure 13: HDM-induced rapid release of IL-33 and glycolytic flux was likely mediated by FPR2 signaling.

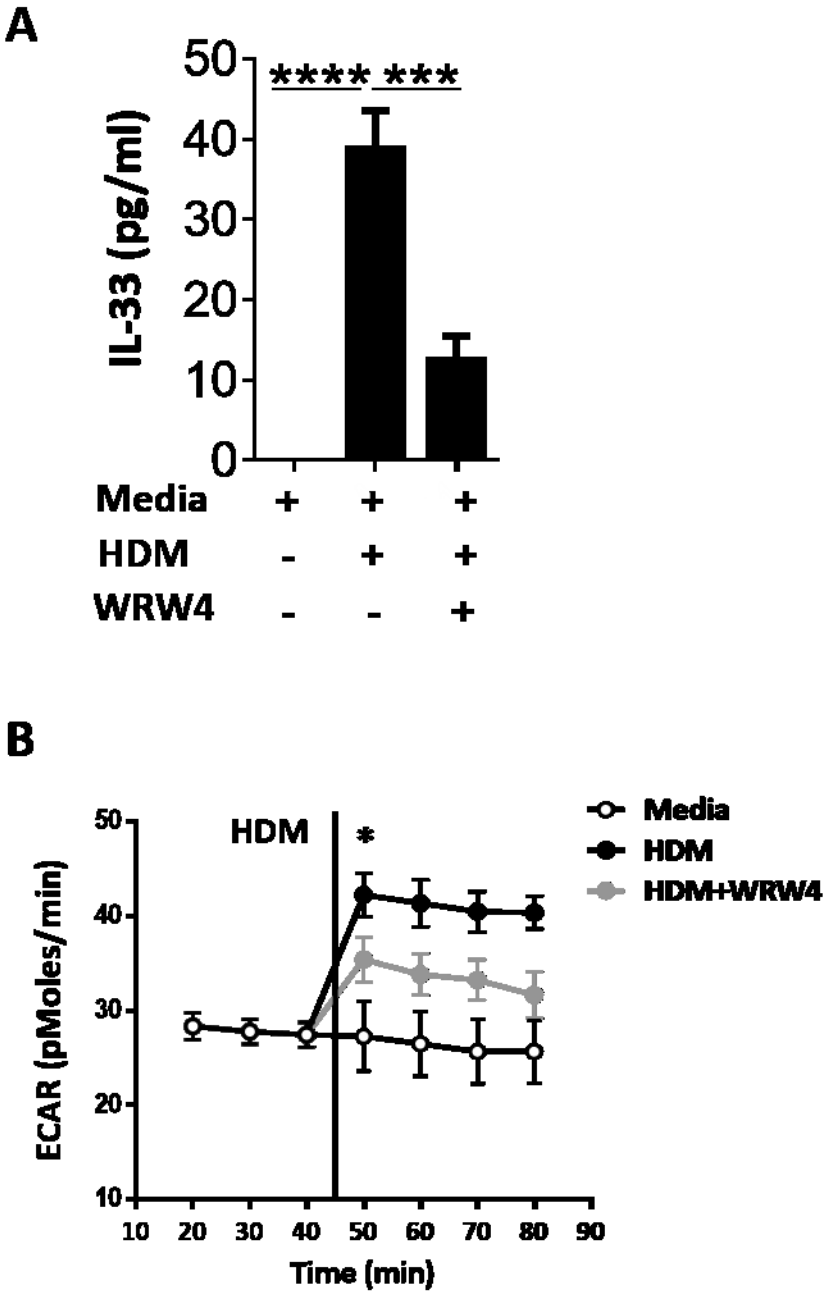
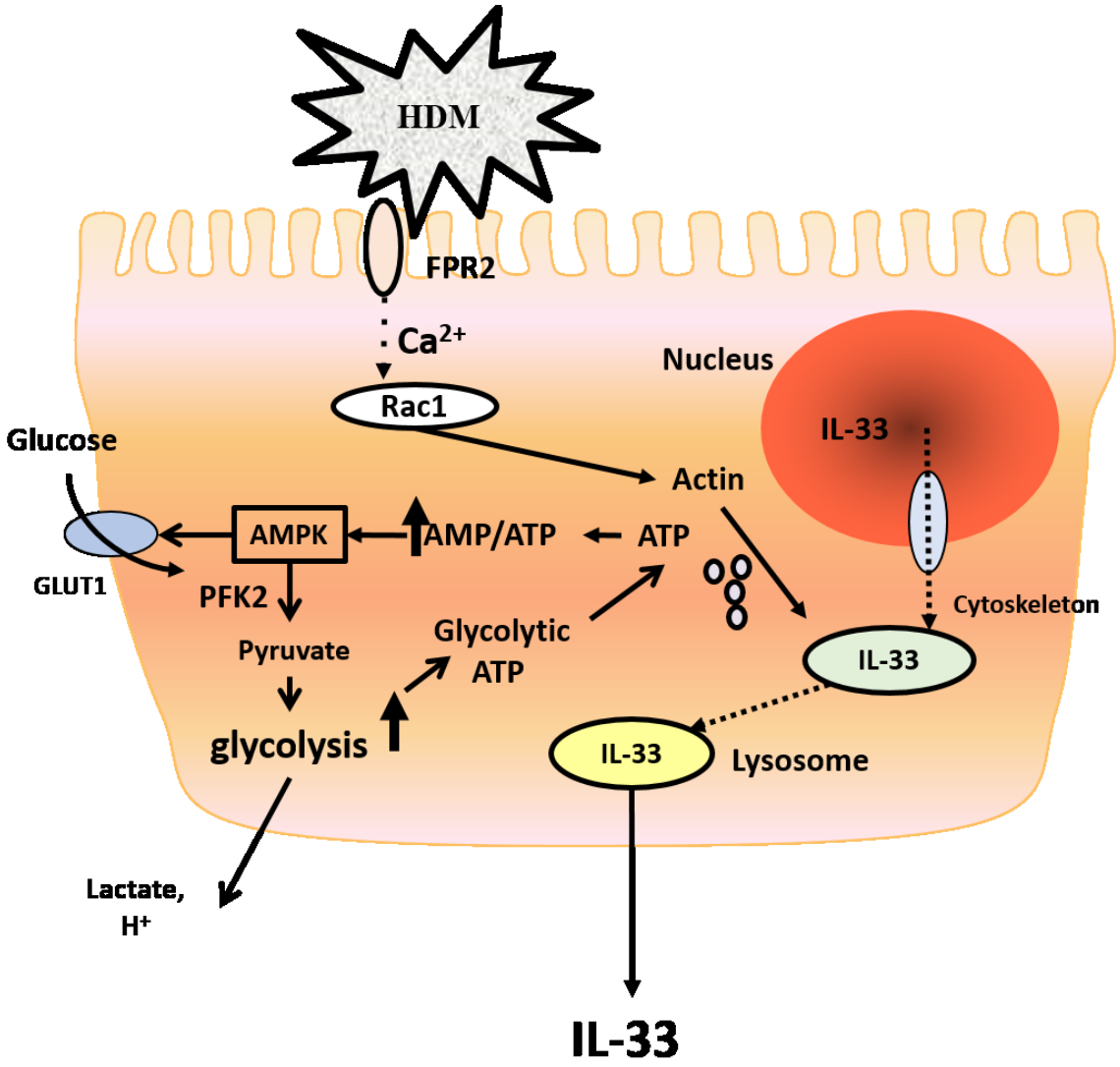


Figure 14: HDM-induced rapid increase of glycolysis facilitates IL-33 release by providing glycolytic ATP. When airway epithelial cells are exposed to HDM, rapid cytoskeleton rearrangements mediated by Rac1 happened for the translocation and release of IL-33. This process rapidly consumed ATP presented in the cytosol, leading to altered AMP/ATP ratio and activation of AMPK signaling. The AMPK then led to an increase of glycolysis probably by promoting glucose uptake and glycolytic enzyme activities. This rapid increase of glycolysis then generates glycolytic ATP to facilitate the early release of IL-33.

Figure 14: HDM-induced rapid increase of glycolysis facilitates IL-33 release by providing glycolytic ATP.



Chapter 5. Changes in cellular metabolism drive HDM-induced allergic asthma *in vivo*.

5.1 Introduction

Based on the data presented in Chapters 3 and 4, in which we saw an immediate induction of glycolytic flux in cultured airway epithelial cells by HDM exposure that led to the release of IL-33, we asked whether allergen-induced alterations in cellular metabolism contributed to the allergic phenotype *in vivo*.

Allergic asthma is thought to arise due to the inappropriate induction of type-2 immune responses against environmental stimuli [455]. Asthma pathology is driven by cytokines associated with this type of immune response, such as IL-4, IL-5 and IL-13 [134]. Of these cytokines, IL-4 is necessary for the development of adaptive Th2 immunity and IgE antibodies against environmental antigens by inducing class switching of the immunoglobulins synthesized by B cells [134]; IL-5 drives eosinophilia in the lung [456]; and most importantly, IL-13 is necessary and sufficient for mounting AHR and excessive mucus production through goblet cell metaplasia [113]. These cytokines are thought to derive mainly from Th2 cells, but numerous recent studies have shown that ILC2s are also potent sources of both IL-5 and IL-13, but not IL-4. Mast cells and basophils are also potential sources of IL-5/IL-13. Together, these findings highlight the importance of understanding the mechanism(s) regulating aberrant type-2 immune responses in the context of allergic asthma.

IL-33, the epithelial cell-produced cytokine [248, 457], has been identified to be a crucial driver of allergic asthma through its ability to promote the secretion of IL-13 and IL-5 from multiple sources such as Th2 cells, ILC2s, as well as eosinophils, basophils, and mast cells [383]. Among these cells, Th2 cells and ILC2s are primary producers of IL-13, the dominant cytokine accounting for allergic asthma pathology [458]. Together, they serve as both early (ILC2) and late (Th2 cells) sources of IL-13 to prime initiation of type-2 immune responses, and to activate effector cells such as airway smooth muscle cells during an asthma attack. The regulatory effects of IL-33 on these cells are mainly through activating its receptor ST2. Specifically, IL-33 activates ST2 on ILC2s to expand the ILC2 population and to induce ILC2 secretion of IL-13 after helminth infection [302]. In mouse models of allergic airway inflammation, IL-33 induces potent type-2 immune responses mediated by early activation of ILC2s [221, 306-308] independently of adaptive immunity [459]. Meanwhile, Th2 cells are found to have inducible *ST2/Il1rl1* expression in the presence of IL-33 and IL-2 [313]. IL-33 itself induces the production of IL-5 and IL-13, but not IL-4, by murine Th2 cells in the presence of antigens [314]. As IL-33 induces IL-13 production by both of these primary sources of IL-13, targeting IL-33 secretion and its resultant ILC2 and/or Th2 cell activation may prove promising for the treatment of allergic asthma.

As IL-33 plays a vital role in directing type-2 immune responses, a recent study also showed that IL-33 is required for HDM-mediated allergic sensitization [460]. We saw a concomitant rapid induction of glycolysis and IL-33 release by HDM in airway epithelial cells, and inhibition of glycolysis with DCA, a glycolysis inhibitor,

diminished IL-33 secretion. These data indicate that cellular metabolism facilitates HDM-induced early release of IL-33 by airway epithelial cells *in vitro*, and whether modulating cell metabolism affects HDM-induced allergic airway inflammation *in vivo* needs to be investigated. Thus we hypothesized that administration of DCA *in vivo* would dampen HDM-induced allergic airway inflammation by reducing airway epithelial release of IL-33, leading to (1) reduced activation of ILC2s or Th2 cells and (2) decreased IL-13 production.

To test this hypothesis, wild-type (WT) BALB/c mice were given either PBS (40 μL) or HDM (100 μg/40 μL) intra-tracheally. In addition, they also received systemic treatment with either H_2O (50 μL) or DCA (5 mg/50 μL) to assess the effects of glycolysis inhibition on allergic inflammation. To understand whether *in vivo* DCA treatment also has effects on effector cells downstream of IL-33 production, the mice were then treated with intra-nasal administration of PBS (50 μL) or recombinant IL-33 (rIL-33) (0.5 μg/50 μL) with and without DCA (5 mg/50 μL) for airway hyperresponsiveness (AHR) measurement.

In the present study, we observed a reduction in HDM-induced early production of IL-33 in the lung following DCA treatment, which was consistent with our *in vitro* findings. DCA also significantly reduced all aspects of allergic airway inflammation, likely via reducing IL-33-dependent production of IL-13 by ILC2s. The fact that DCA had no effect on AHR induced by rIL-33 suggested that it did not affect the effector cells directed by IL-33, confirming that modulating glucose metabolism likely affected mainly IL-33 production and resultant allergic airway inflammation.

5.2 Results

5.2.1 Identification of *in vivo* effect and kinetics of DCA.

As a glycolysis inhibitor utilized for the treatment of lactic acidosis [461] and cancer [462], DCA was recently also found to modulate cell metabolism and ragweed-induced allergic airway inflammation in mice [370]. To verify our observation that DCA reduced HDM-induced IL-33 release *in vitro*, we sought to first determine the appropriate dose and timing of DCA treatment *in vivo*. To this end, wild type BALB/c mice were given an intra-peritoneal injection of H_2O (50 μL) or DCA (5 mg/50 μL or 10 mg/50 μL). Cheek blood was then collected from these mice using serum gel separator tubes at 1 hour, 4 hours and 24 hours after DCA treatment. The serum was then separated for determination of the levels of the glycolysis end product lactate. 5 mg/50 μL DCA significantly reduced the level of lactate in the serum compared to controls at 1 hour post-treatment (Fig. 15A). The reduction of lactate was dampened at 4 hours post-treatment (Fig. 15B), and disappeared at 24 hours (Fig. 15C). Notably, 10 mg/50 μL DCA did not reduce the lactate in the serum, but rather tended to increase lactate at 4 hours (Fig. 15B) and 24 hours (Fig. 15C) post-treatment.

5.2.2 HDM-induced increase in anaerobic glycolytic flux was associated with IL-33 secretion *in vivo*.

We then examined whether HDM exposure induced glycolysis and IL-33 release in the airways *in vivo*. As IL-33 was induced by HDM within 30 minutes *in vitro*, we assessed the release of IL-33 into the airways after HDM exposure in mice

in which glycolysis was inhibited by treatment with DCA. According to the range finding experiments discussed in 5.2.1, we identified 5 mg to be an appropriate dose of DCA to inhibit glycolysis. The relatively rapid dampening of effects indicated a short half-life of DCA *in vivo*. So we designed the DCA treatment regimen to include treatment of mice 1 hour prior to as well as every 8 hours after each HDM challenge (Fig. 16A). WT BALB/c mice were administered 40 μL PBS or 100 μg/40 μL HDM intra-tracheally with and without intra-peritoneal injection of DCA, and BAL was collected 18 hours later (Fig. 16A). We observed that within 18 hours, HDM-induced a significant increase in BAL IL-33 levels, which were reduced by DCA treatment (Fig. 16B). Lactate production was induced by HDM, and inhibited by DCA (Fig. 16C). These data supported the hypothesis that HDM induced increases in glycolysis were associated with the induction of IL-33 release in the airways *in vivo*.

5.2.3 Inhibition of glycolytic flux reduced HDM-induced allergic airway response.

To determine whether DCA-induced suppression of IL-33 was associated with alterations in the pathological aspects of asthma, we compared allergic lung inflammation in HDM-challenged WT BALB/c mice with and without DCA treatment. Specifically, mice received 2 doses of 100 μg/40 μL HDM intra-tracheally on day 0 and day 14 according to the previously developed protocol [193]. To modulate the early glucose metabolism alterations in airway epithelial cells by HDM, 5 mg/50 μL DCA was given intra-peritoneally 1 hour prior to and once every 8 hours within the first 72 hours after each HDM treatment (Fig. 17A). 72 hours after

the last HDM challenge, mice were sacrificed to assess allergic airway responses. Increases in airway pressure in response to cholinergic agonist (methacholine) stimulation (assessed by airway pressure time index, APTI) were significantly higher in mice with HDM challenge than PBS controls, while DCA treatment significantly reduced airway responsiveness (Fig. 17B). Mice receiving HDM had significantly higher levels of IgE in the serum compared with those receiving PBS, and DCA treatment reduced the IgE production (Fig. 17C). HDM treatment caused eosinophilia in these mice, another hallmark of allergic airway inflammation, which was reduced by DCA treatment (Fig. 17D). In contrast, while HDM exposure also recruited neutrophils to the lung, DCA treatment further enhanced the infiltration of neutrophils (Fig. 17E). We also assessed the airway mucus production by measuring the expression of *Muc5ac* and *Muc5b*, the genes encoding mucins produced in significant quantities in human and mouse airways [463]. As expected, HDM significantly increased their expression compared to controls, indicating increased mucus production in the airways. However, the mucin gene expression was reduced by DCA treatment (Fig. 17F – G). To visualize the thickening and structural changes within the airways, we assessed the morphology of the airways by staining lung sections with hematoxylin and eosin. We saw that while HDM treatment caused obvious thickening and structural changes in the airways, DCA treatment revealed significant amelioration of the pathological alterations (Fig. 17H). Despite the effect of DCA on the classical type-2-cytokine-mediated features of asthma, flow cytometric analysis of Th2 cell prevalence (IL-13+CD4+TCRβ+) did not show a significant reduction in DCA-treated mice as compared to those with HDM-

treatment alone (Fig. 17I), and the expression of *Il13* in the lung was also not significantly affected by DCA at this time point (Fig. 17J).

5.2.4. DCA reduced early ILC2 recruitment and BAL IL-13 levels.

To evaluate whether earlier changes in type-2 cytokines were involved and played a role, WT BALB/c mice were treated with DCA and HDM following the protocol shown in Fig. 16A. At the end of the experiment, the total lung tissues of mice were harvested for flow cytometric analysis of lung ILC2 prevalence (Lin-CD45+ST2+IL-13+). IL-13 levels in the BAL were also measured. Using the gating strategy shown in Fig. 18A, we found a significant increase in ILC2 prevalence in the lung after HDM exposure accompanied with IL-13 production compared to PBS controls (Fig. 18B – C), consistent with the literature that ILC2s are recruited in the presence of IL-33 during allergic inflammation as a relatively small cell population but major producers of IL-13 [301]. Consistent with our earlier findings, DCA administration to mice *in vivo* significantly reduced both ILC2 recruitment and their IL-13 production induced by HDM treatment (Fig. 18B – C). Taken together with the data presented in Fig. 16, our results supported the hypothesis that HDM-induced changes in cellular metabolism lead to IL-33 release, ILC2 recruitment and production of type-2 cytokines, and subsequent development of allergic inflammation.

5.2.5. HDM-induced glycolysis did not influence responsiveness to IL-33 during allergic asthma pathogenesis.

Although our results suggested that glycolysis regulated IL-33 release, it was also possible that glycolysis might regulate processes downstream of IL-33 production. To explore this possibility, we administered recombinant IL-33 (rIL-33) into the mouse airways to induce allergic airway responses, and analyzed the effect of DCA treatment. Specifically, WT BALB/c mice received intra-nasal administrations of rIL-33 (0.5 μg/50 μL) on day 0, 3 and 6; control mice received 50 μL PBS at each time point. Some mice were also given intra-peritoneal injection of DCA (5 mg/50 μL) 1 hour prior to and once every 8 hours after rIL-33 administration. Mice were sacrificed to assess allergic airway responses on day 7 (Fig. 19A). The airway responses to methacholine stimulation were significantly higher in mice with rIL-33 challenge than PBS controls, while DCA treatment did not affect the AHR (Fig. 19B). We then analyzed the prevalence of Th2 cells and ILC2s. rIL-33 caused increased recruitment of these cells to the lung, but DCA treatment had no effect (Fig. 19C – D). Consistently, rIL-33 induced significant increases in IL-13 production in the lungs as compared with those in saline-treated control mice, but little modulation of IL-13 was seen by DCA treatment (Fig. 19E). Together these data suggested that modulating glucose metabolism had little effect on the responsiveness of lung cells to IL-33. These data support our contention that allergen-induced changes in metabolism affect allergic inflammation likely through the regulation of IL-33, not downstream cellular responses to IL-33.

5.3. Discussion

Based on our previous *in vitro* finding that HDM induced a rapid increase in glycolysis in airway epithelial cells to facilitate the release of IL-33, we sought to determine whether such metabolic changes contributed to the allergic phenotype *in vivo*. Consistent with our *in vitro* observation, HDM caused increases in glycolysis in the lungs of allergen-exposed mice, concomitant with elevations in the IL-33 levels in the airway. Our data demonstrate that short-term exposure to HDM leads to a significant elevation of *in vivo* lung lactate levels (Fig. 16C). This is consistent with the previous publication that early asthma-initiating responses in allergen-sensitized rats are associated with increased glycolytic activities [371], suggesting the correlation between initiation of allergic inflammation and glycolysis. When mice also received DCA, the enhanced glycolysis was reversed in the lung, consistent with a previous report that *in vivo* administration of DCA could reduce glycolytic activities in the mouse lung after ragweed challenge [370]. We also saw an early increase in IL-33 levels in the lung (Fig. 16B). This is consistent with the recent report showing the early induction of IL-33 *in vivo* by HDM exposure [245], highlighting early IL-33 release as an essential mechanism by which HDM induces allergic airway inflammation. The fact that modulating glycolysis with DCA also reduced IL-33 release confirmed our *in vitro* observation that HDM-induced early increase of glycolysis was required for IL-33 secretion, as well as the novel mechanism that glycolysis facilitated IL-33 release. Together, these results support the hypothesis that HDM induces increases in glycolysis, which are associated with IL-33 production *in vivo*.

However, we do not have direct evidence that airway epithelial cells are the primary sources of either lactate or IL-33 in the lung. Both lactate and IL-33 can be produced by multiple other cells including DCs [464], macrophages/monocytes [465-467], mast cells [274, 468] and neutrophils [469] that play a role in allergic asthma. Although no evidence to date has shown that HDM can induce lactate and IL-33 release by these cells at this time point, our data does not rule out the contributions of these cells. As a result, direct analysis of the sources of lactate and IL-33 at this time is needed. Since we believe that IL-33 live cell secretion is mediated by translocation, analyzing the cell type(s) in the airway in which intracellular IL-33 is reduced after HDM exposure will provide the answer. To do this, future studies utilizing IL-33 reporter mice to determine the location of IL-33-producing cells in lung biopsies after HDM treatment with and without DCA are needed. Direct evidence for the source of lactate might be harder to obtain as lactic acid is produced by every cell type in the lung. However, techniques using fluorescent glucose analogues allow *in vivo* monitoring of glucose by positron emission tomography (PET) imaging and near-infrared spectroscopy (NIRS) [470, 471].

The current study also failed to show that DCA blockade of glycolysis mainly affected airway epithelial cells, as systemic DCA administration was likely affecting all the cells. However, targeting glycolysis in specific tissues/cells remains difficult. For example, pharmacological inhibition of cell metabolism sometimes results in activation of compensatory mechanisms, which are likely the case in the current study as we observed that a higher dose of DCA actually caused increased serum

lactate (Fig. 15). Also, mice with genetic deficiency of essential glucose metabolism regulators often die as neonates. As a result, the best approach to inhibit glycolysis in airway epithelial cells might be to use shRNA designed to express specifically in the airway epithelial cells to knock down PDK1 (the target of DCA) or LDH (the target of oxamate) to moderately reduce glycolytic activity in these cells.

To determine whether allergen-induced changes in metabolism played a role in the allergic phenotype *in vivo*, we explored the effect of DCA treatment on allergic airway inflammation. We observed a reduction in allergic airway responses including AHR, eosinophilia, IgE antibody production, mucus production and airway structural changes when HDM-induced glycolysis was inhibited (Fig. 17A – H). Our studies demonstrate that HDM-induced allergic airway inflammation was reduced by DCA, and is consistent with the previous report that DCA treatment reduced airway reactivity to methacholine and mucus production induced by ragweed [370]. Moreover, the fact that almost all aspects of allergic inflammation were reduced by DCA suggested that it diminished the initiation of HDM-induced allergic asthma, supporting the importance of cell metabolism in the early phase of allergic asthma pathogenesis.

As IL-13 is the central mediator of allergic airway inflammation, it was expected that DCA treatment inhibited IL-13 production. However, although there seemed to be slightly fewer Th2 cells and less *Il13* expression in the lungs of DCA-treated mice (Fig. 17I – J), such moderate suppression could not explain the significantly reduced pathological features. It was also inconsistent with the previous report that DCA reduced IL-13 in a ragweed-induced experimental asthma

model [370]. However, there are differences in the compositions [472, 473] of ragweed and HDM, indicating that distinct pathways may be induced, especially since ragweed has not been shown to induce IL-33 in the airway epithelial cells. Also, DCA administration in the ragweed study occurred during the effector phase of the response, not at initiation, while in our model it was given both at the time of sensitization and challenge, with a focus on the first 72 hours post-HDM exposure. The difference in the timing of the DCA treatment suggested that DCA given in the ragweed study probably conferred major effects on CD4+ T cells by inhibition of their proliferation and cytokine secretion. Their findings were consistent with the idea that activated T cells depend on glycolysis for secretion of cytokines [474]. In our study DCA probably mainly affected another earlier source of IL-13. Considering that another major and earlier source of IL-13 is ILC2, the innate immune cells that are recruited by IL-33 and prime HDM-induced type-2 immune responses [221, 475], we hypothesized that ILC2 recruitment and production of IL-13 were affected by DCA treatment.

Indeed, we showed that the recruitment of ILC2s into the lungs after HDM exposure was significantly reduced by DCA treatment (Fig. 18). These data suggested that DCA significantly reduced the early source of IL-13, which provided evidence that the reduced allergic airway inflammation might be due, in part, to the reduction of the earlier effects of IL-13. In fact, a recent study examined the allergic airway inflammation induced by HDM in mice with genetic depletion of ILC2s (RORα -/-) and found that lung ILC2s played a critical role in priming the adaptive type-2 immune response including the recruitment of eosinophils and serum IgE levels.

Although their study did not examine the role of ILC2s in AHR or Th2 cytokines production in the lung [475], the results suggested that Th2 cytokines derived from ILC2s were critical for the development of allergic responses. These results support our hypothesis that modulating earlier ILC2 production of IL-13 affects allergic asthma development.

Our data provide strong evidence that modulating glucose metabolism alters IL-33 production after HDM exposure, interferring with the progression of HDM-induced allergic asthma via inhibition of ILC2-mediated priming of type-2 immune response. However, several limitations of the current studies should be considered. First, the importance of ILC2-derived Th2 cytokines in the development of AHR still remains to be determined. To do this, follow up studies examining AHR induced by HDM in mice deficient of ILC2 will be necessary. Also, we propose that IL-13 derived from both ILC2s and Th2 cells likely contributes to allergic asthma pathogenesis at different time points, but limited evidence is available yet for the detailed kinetics of IL-13 production *in vivo* following HDM exposure. To prove our hypothesis, defining the temporal pattern of IL- 13 production *in vivo* following HDM exposure would be necessary. More importantly, as discussed earlier, DCA was given intra-peritoneally in this mouse model of experimental asthma. Such systemic administration affects not only airway epithelial cells, but also other cells presented at the time of DCA treatment. In fact, it was published that B cell activities were dependent on glycolysis, and DCA treatment sharply suppressed B cell proliferation and antibody secretion *in vitro* and *in vivo* [374]. Consistent with these concepts, the reduction of IgE we observed in DCA-treated mice in the current study could be

attributed to the effects of DCA on B cells. Moreover, whether DCA also affected other cells that contribute to allergic airway responses remains unknown, and future studies on the effects of specific knockdowns of genes regulating glycolysis in epithelial cells on HDM-induced AHR are required.

Another observation worth further attention was the significantly increased neutrophil recruitment in mice treated with DCA (Fig. 17E). Our colleagues have shown that depletion of neutrophils with Ly6G following 24 hours HDM exposure resulted in significantly elevated IL-8, slightly increased IL-33 and IL-13 in the BAL, as well as more ILC2 recruitment to the lung comparing to mice receiving only HDM (data not published). These data are consistent with our observation that inhibition of IL-33 and early ILC2 activation by HDM occurred concomitantly with the increased neutrophilia, suggesting that neutrophils recruited to the lung at this time may confer protective effects against allergic inflammation. However, the role of neutrophils in allergic asthma remains unclear [476]. Whether the increased neutrophils in response to glycolysis inhibition protect against HDM-induced allergic asthma, and how inhibition of glycolysis results in increased recruitment of neutrophils are questions remain to be studied.

Since the current *in vivo* administration of DCA might affect multiple cell types involved in allergic asthma pathogenesis, we also attempted to understand whether its reduction of allergic airway inflammation was attributed to its actions on effector cells downstream of IL-33 by analyzing the effects of DCA on AHR induced by rIL-33. Our observation that rIL-33–induced AHR, ILC2s, Th2 cells, and IL-13 production in the lung were not affected by DCA treatment (Fig. 19) indicated

that DCA did not affect the activities of effector cells downstream of IL-33 in allergic airway responses. These results might seem inconsistent with the earlier ragweed study with DCA, in which DCA was thought to inhibit CD4+ T cell proliferation [370]. However, no direct evidence for DCA inhibition of Th2 cells *in vivo* was presented in the ragweed study. Moreover, ILC2s are likely the major cell type responding to IL-33 in our model, which is confirmed by the fact that the prevalence of Th2 cells in these mice was very low. These data further indicated that Th2 cells probably played only a limited role in this model. Taken together, these data are consistent with the literature that IL-33-induced ILC2s are probably sufficient to induce allergic airway responses [459] and that allergen-driven changes in metabolism likely induce the allergic phenotype through regulating the production of IL-33, rather than altering downstream responsiveness to IL-33.

In conclusion, we demonstrate a novel mechanism by which HDM induces the rapid increase of glycolysis in airway epithelial cells to facilitate IL-33 release, leading to the recruitment and activation of ILC2s and the development of allergic asthma. The current study reproduced our *in vitro* observation and proved the importance of this mechanism in asthma pathogenesis *in vivo*. While further studies addressing the discussed caveats are needed, we conclude that blocking glycolysis likely reduces IL-33 release and resultant allergic airway inflammation *in vivo*. As a result, local instillation or systemic ingestion of glycolysis inhibitors such as DCA may provide a successful strategy to either prevent or suppress asthma symptoms. Moreover, administration of DCA earlier in life may serve as a novel preventive approach for susceptible individuals.

5.4 Figures

Figure 15: Identification of *in vivo* effect and kinetics of DCA. To determine the optimal dose and timeframe for *in vivo* blockade of glycolysis with DCA, WT BALB/c mice were given intra-peritoneal injection of PBS (50 µL) or DCA (5 mg/50 µL and 10 mg/50 µL). Cheek blood was collected using serum gel separator tubes at (A) 1 hour, (B) 4 hours and (C) 24 hours after DCA treatment from these mice. The serum was then separated, and levels of glycolysis end product lactate were measured by colormetric assays. Data presented as mean values ± SEM for biological replicates n=3, *$p<0.05$ (one-way ANOVA and Dunn's post test).

Figure 15: Identification of *in vivo* effect and kinetics of DCA.

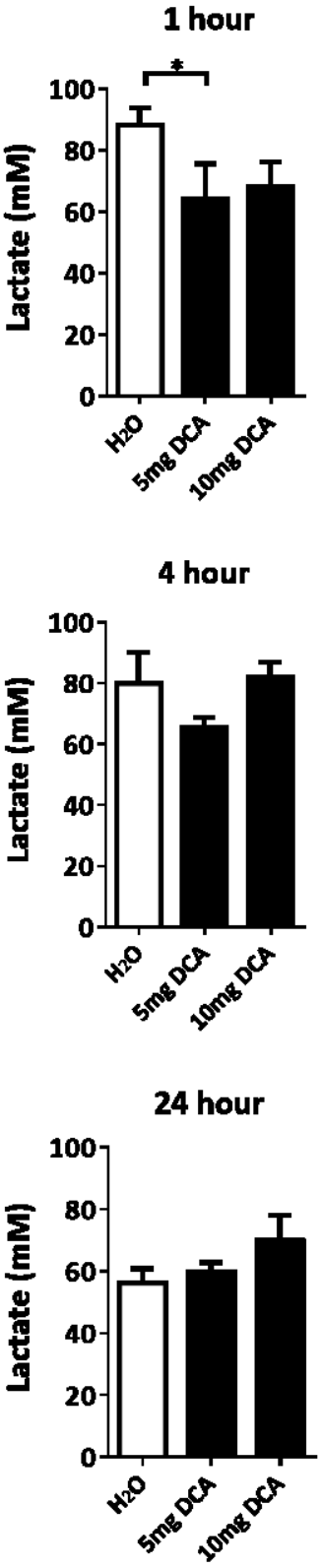


Figure 16: HDM-induced increases in anaerobic glycolytic flux was associated with IL-33 secretion *in vivo*. WT BALB/c mice were given a single dose of intra-tracheal PBS (40 μL) or HDM (100 μg/40 μL) to induce early production of IL-33 in the lung. To inhibit early induction of anaerobic glycolysis, these mice were further grouped to receive intra-peritoneal injection of H_2O (50 μL) or DCA (5 mg/50 μL) 1 hour prior to and every 8 hours after HDM treatment (A). After 18 s of exposure to HDM, BAL was collected for assessment of (B) IL-33 secretion and (C) lactate production. Data presented as mean values ± SEM for biological replicates n=4-5, *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA and Dunn's post test).

Figure 16: HDM-induced increases in anaerobic glycolytic flux were associated with IL-33 secretion *in vivo*.

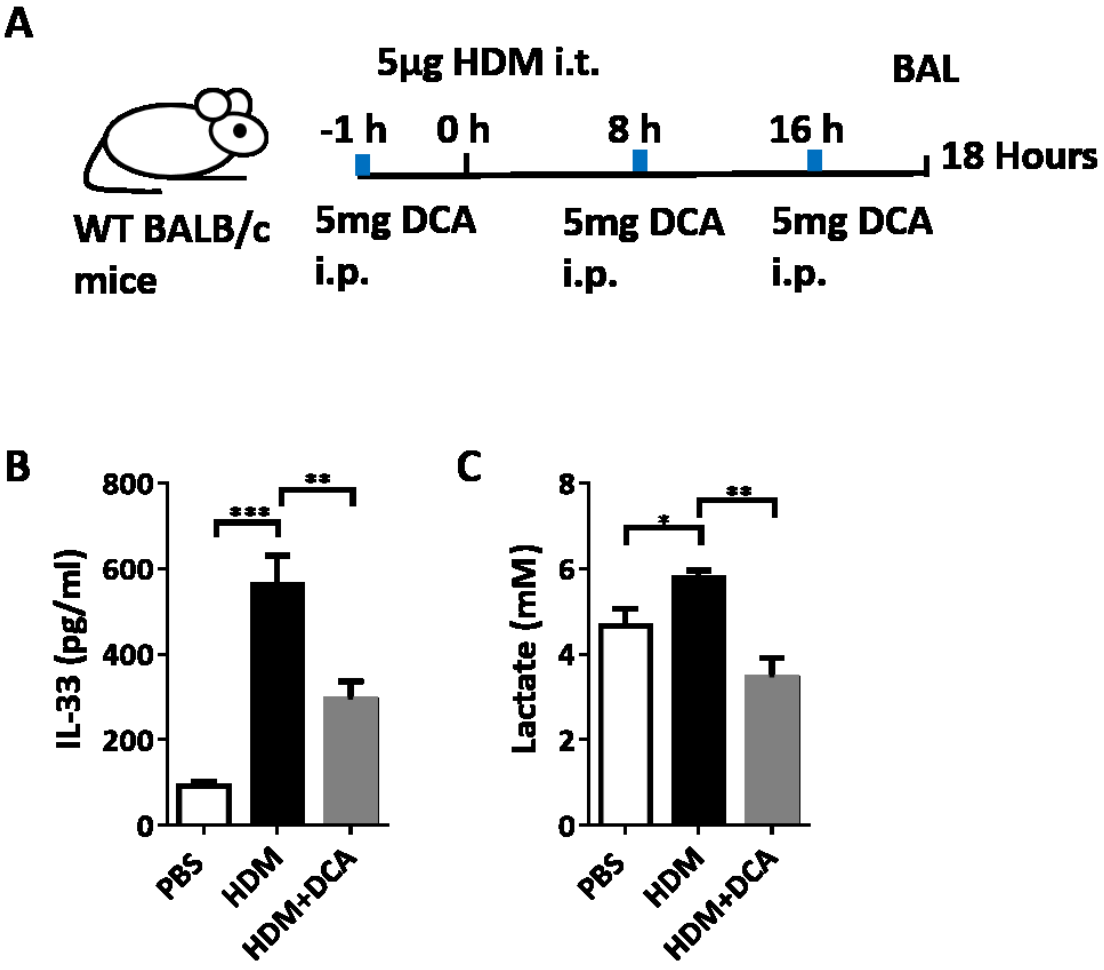


Figure 17: Inhibition of glycolytic flux reduced HDM-induced allergic airway response. WT BALB/c mice were given intra-tracheal PBS (40 μL) or HDM (100 μg/40 μL) on day 0 and day 14 to mount the allergic asthma phenotype. They were further grouped to received intra-peritoneal injection of DCA (5 mg/50 μL) during the phase of early responses to each HDM exposure 1 hour prior to and once over 8 hours after HDM treatment for 72 hours. The treatment regimen is shown in (A). 72 hours after the last HDM treatment, mice were euthanized and HDM-induced allergic airway response was analyzed by measuring (B) AHR. (C) Serum IgE was measured using ELISA. BAL (D) eosinophils and (E) neutrophils were identified with May-Grünwald Giemsa staining and counted. Expression of epithelial mucin genes (F) *Muc5ac* and (G) *Muc5b* was determined by real-time PCR. (H) Lung sections (three per experimental group and three sections per animal) were fixed in formalin; cut into 10-μm sections; and stained with hematoxylin & eosin for histology analysis. Representative sections are shown. Part of the lung tissue was processed to make single cell suspension for flow-cytometric analysis of (I) Th2 cell prevalence, and (J) *Il13* expression in the lung was determined by real-time PCR. Data presented as mean values ± SEM for biological replicates n=7-8, *$p<0.05$, **$p<0.01$, ****$p<0.0001$ (one-way ANOVA and Dunn's post test).

Figure 17: Inhibition of glycolytic flux reduced HDM-induced allergic airway response.

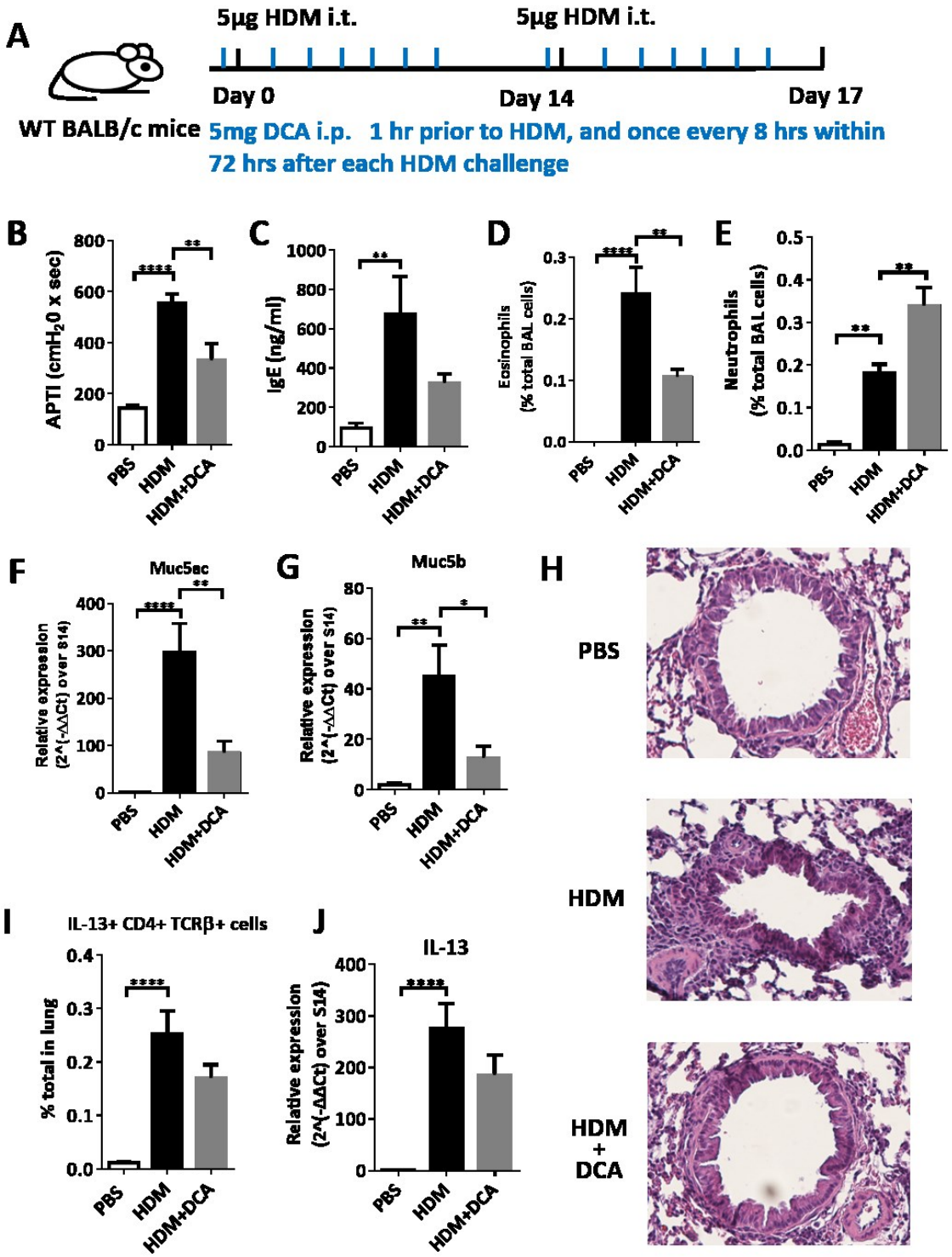


Figure 18: DCA reduced early ILC2 recruitment and BAL IL-13 levels. WT BALB/c mice were challenged intra-tracheally with PBS (40 μL) or HDM (100 μg/40 μL) and treated with H_2O (50 μL) or DCA (5 mg/50 μL) following the protocol shown in Fig. 16A. BAL was collected; mouse lung was harvested and processed into single cell suspension for flow-cytometry analysis. (A) ILC2 was gated as Lin-CD45+ST2+IL-13+. (B) Prevalence of ILC2s recruited to the lung was determined, and (C) IL-13 level in the BAL was measured with ELISA. Data presented as mean values ± SEM for biological replicates n=4-5, *p<0.05, ***p<0.001, ****p<0.0001 (one-way ANOVA and Dunn's post test).

Figure 18: DCA reduced early ILC2 recruitment and BAL IL-13 levels.

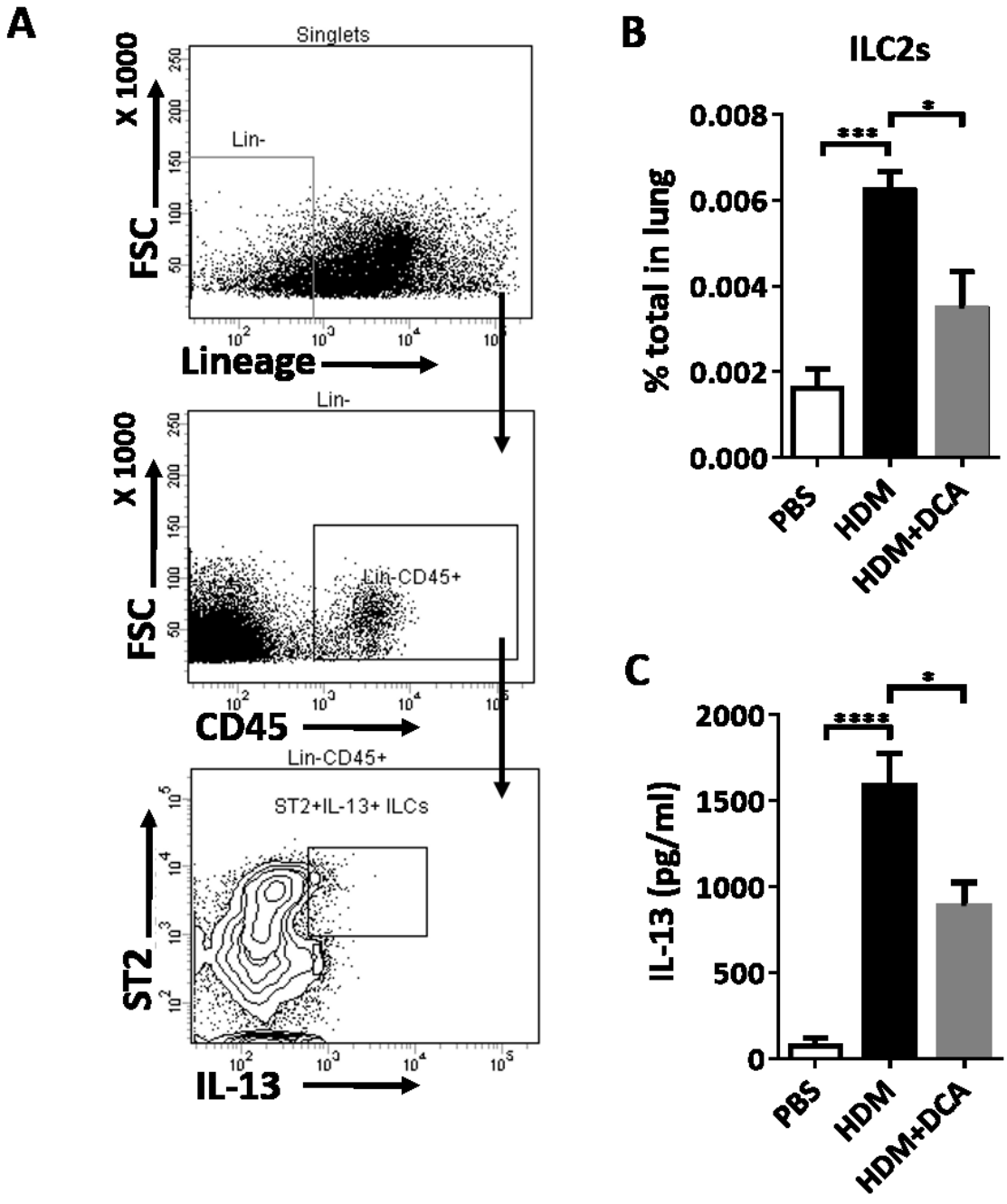
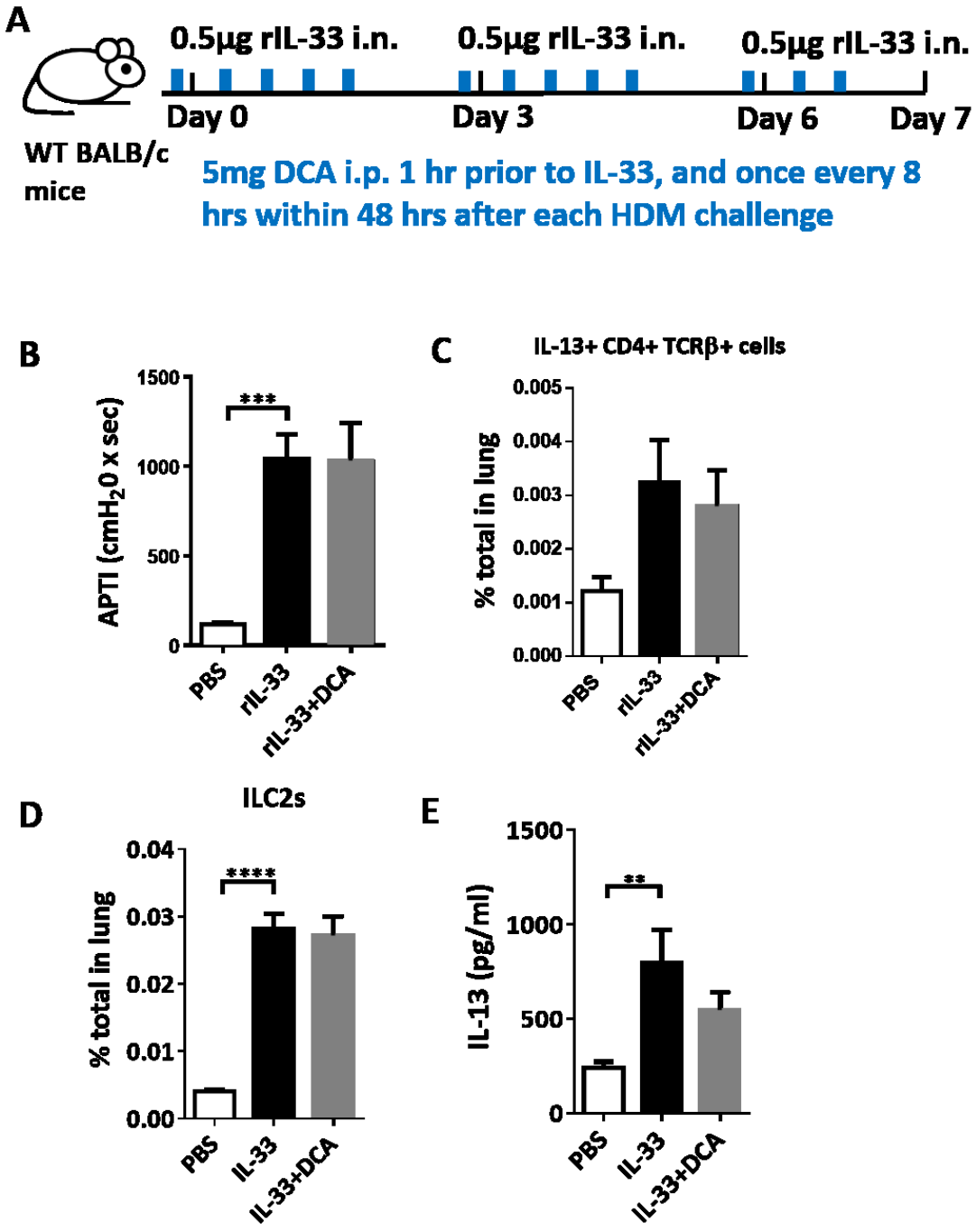


Figure 19: HDM-induced glycolysis did not influence responsiveness to IL-33 during allergic asthma pathogenesis. WT BALB/c mice were treated intra-nasally with rIL-33 to elicit experimental allergic asthma. The mice received either PBS (50 μL) or rIL-33 (0.5 μg/50 μL) on day 0, 3 and 6. The mice were then grouped to receive H_2O (50 μL) or DCA (5 mg/50 μL) treatment 1 hour prior to and once every 8 hours after rIL-33 administration for 48 hours as shown in (A). 24 hours after the last rIL-33 treatment, mice were euthanized and rIL-33 induced airway responses was analyzed by measuring (B) AHR. Lung tissues were harvested and processed into single cell suspension for flow-cytometric analysis of (C) Th2 cell and (D) ILC2 frequencies, and (E) IL-13 level in the BAL was determined by ELISA. Data presented as mean values ± SEM for biological replicates n=6, **p<0.01, ***p<0.001, ****p<0.0001 (one-way ANOVA and Dunn's post test).

Figure 19: HDM-induced glycolysis did not influence responsiveness to IL-33 during allergic asthma pathogenesis.



Chapter 6: Discussion and future research.

6.1 Summary

Allergic asthma is a major public health problem affecting approximately 300 million people worldwide [5]. Currently, individuals suffering from asthma primarily receive corticosteroids and β-agonists for immunosuppression and bronchodilation during an asthma attack [477]. However, these therapies are not effective in many asthmatics, and generally only offer temporary relief from the symptoms, rather than modifying the course of disease [478]. As a result, understanding the factors driving allergic asthma in susceptible individuals may lead to the development of more effective therapeutic approaches that might modify the natural history of the disease in all asthmatics.

During the past few decades, our understanding of the pathogenesis of asthma has significantly increased. Substantial evidence suggests that aberrant type-2 immune responses to ubiquitous aeroallergens drive disease pathology in genetically susceptible individuals. Although the factors leading to such type-2 cytokine skewed immune responses in asthma are not well understood, recent evidence suggests that elevated secretion of the type-2 promoting cytokine IL-33 from the airway epithelium plays an important role in the initiation of the disease. Indeed, several lines of evidence support a major role for IL-33 in allergic asthma including the association of SNPs in *Il33* and its receptor *ST2/Il1rl1* with risk of developing asthma, elevated levels of *Il33* expression in lung tissues from asthmatics, and the growing number of studies in experimental models of asthma

demonstrating the critical importance of IL-33 and ST2 in the development of the allergic airway inflammation [240, 382].

Despite strong evidence for the role of IL-33 in asthma susceptibility, little is known about its induction, especially by environmental stimuli such as allergens. Recent studies suggest that allergens may regulate the rapid release of IL-33 from airway epithelial cells [245, 249, 460] independently of effects on gene transcription. Although it has been postulated that allergens containing proteases may induce IL-33 release from the airway epithelium through activation of oxidative stress pathways, it is unclear whether all allergens elicit IL-33 release through a common mechanism involving proteases.

The studies reported in this thesis have identified a novel mechanism by which the common allergen, HDM, causes the rapid increase of glycolysis in cultured airway epithelial cells, which is associated with the early release of IL-33 (Chapter 3). Taken one step further, we provide substantial new information regarding the mechanisms of allergen-driven IL-33 release from epithelial cells and the pathways by which glycolysis contributes to this process. We propose that by recognizing certain components in HDM, the formyl peptide receptor 2 (FPR2) likely causes increased intracellular calcium flux and activation of Rac1, which activates cytoskeleton rearrangement and IL-33 release.

As this process consumes large amounts of ATP, energy deprivation will lead to activation of AMPK to induce glycolytic flux, which in turn fuels the IL-33 secretion process through the production of glycolytic ATP (Chapter 4). To further understand the significance of this mechanism in allergic asthma pathogenesis, we

utilize an *in vivo* model of experimental asthma and demonstrate that inhibition of anaerobic glycolysis reduces airway epithelial cell release of IL-33 upon HDM exposure, which dampens the early recruitment and activation of ILC2s and diminishes allergic airway inflammation (Chapter 5). These *in vivo* data add considerable support for the concept that HDM regulation of cellular glycolytic pathways plays a pivotal role in IL-33 release and allergic asthma pathogenesis.

Taken together, we have identified a novel mechanism by which HDM induces IL-33 release and the initiation of allergic asthma (Fig. 20). We confirm that HDM causes the rapid release of IL-33 independent of cell death or *de novo* protein synthesis and discover that allergen-driven changes in cellular glycolytic flux and glycolytic ATP facilitate IL-33 release. These studies have highlighted a previously understudied area-glucose metabolism-as an important direction for future study of allergic asthma pathogenesis. As the role of cellular glucose metabolic pathways have been recently revisited in many diseases and conditions such as cancer, our study provides additional evidence for its importance in human health.

6.2 Caveats and future directions

Despite the advances in our knowledge of the mechanisms regulating IL-33 release by airway epithelial cells reported in this thesis, several unanswered questions remain to be addressed. In the remainder of this chapter, we will discuss the caveats of our studies, the future directions of the current research, and the broader implications of our findings for human allergic disease.

6.2.1 Detailed phenotyping of HDM-induced glycolytic flux is needed.

HDM exposure was found to induce a rapid increase in the glycolytic flux in airway epithelial cells. However, little is known about the long-term effects of elevated glycolysis on the airway epithelial phenotype. Recent studies have shown that PRR ligands induce different immediate and chronic metabolic alterations that play different roles in cells. For example, in bone marrow-derived DCs, researchers have shown that LPS causes a rapid induction of glycolytic flux, but no suppression of OXPHOS, to mediate DC activation [338]. However, exposure for periods longer than 12 hours causes the cells to utilize Warburg metabolism, a phenomenon in which cells rely mostly on glycolysis for glucose metabolism for their bioenergetics demand while OXPHOS is largely inhibited [337]. This begs the question of whether similar mechanisms exist in airway epithelial cells. If so, could these metabolic alterations be associated with the remodeling of the airway? In fact, studies have found that prolonged exposure to HDM resulted in an epithelial-to-mesenchymal transition in human bronchial epithelium [479], a process well known to involve glucose metabolic changes [480, 481]. In our studies, we have observed significant airway remodeling in the mouse lung after HDM exposure, which was significantly ameliorated by DCA treatment, supporting the hypothesis that modulating cell metabolism may affect the airway remodeling process. Taken together, examination of the long-term effects of allergen-induced alterations in glucose metabolism on epithelial function may further advance our understanding of disease pathogenesis.

6.2.2 The potential component(s) in HDM that induces IL-33 release and glycolysis needs to be identified.

Our data strongly suggest that the G-protein coupled receptor FPR2 recognizes certain components of HDM and induces IL-33 release and glycolysis. Our data support a scenario in which FPR2 activation leads to increased intracellular Ca^{2+} levels and subsequent activation of cytoskeleton rearrangement, leading to IL-33 translocation from the nucleus and secretion from the cells. However, direct evidence for this link is not provided in our studies. Studies designed to monitor (1) changes of intracellular Ca^{2+} fluctuation as well as (2) Rac1 activation and cytoskeleton rearrangement upon HDM exposure with and without FPR2 inhibition are needed to definitively prove this link. Moreover, even if we prove such a link, the potential ligands in HDM that activate FPR2 remain to be identified. FPR2 is widely recognized to trigger both pro- and anti-inflammatory responses depending on the nature of the ligands [416, 450], which can be either host-derived endogenous molecules or exogenous substrates. It has different receptor domains that recognize lipid and peptide/protein ligands [449, 450], such as lipid mediator lipoxin A_4 (LXA4) and the pro-Th2/Th17 acute phase protein SAA [451-454]. Thus, we hypothesize that HDM may contain FPR2 ligands or that HDM induces immediate host production of the ligands by epithelial cells, leading to autocrine actions on the epithelium and IL-33 release. Our current data suggest that blocking the peptide-binding domain with WRW4 abrogated HDM induced IL-33 release and glycolytic flux. However, such inhibition may affect the conformation of FPR2 and the binding to both protein and lipid ligands. As a result, the current data do not provide hints

on the possible FPR2 ligands in HDM. To understand this, direct mass spectrometry analysis of HDM components should reveal the presence of FPR2 ligands or structurally similar substances. If we find no direct evidence for FPR2 ligands in HDM, the culture supernatants of airway epithelial cells exposed to HDM should be analyzed to determine whether HDM induces release of FPR2 ligands for its autocrine activation.

6.2.3 The exact mechanisms of IL-33 release remain to be elucidated.

In addition to the need for a more detailed understanding of the mechanisms by which HDM induces metabolic alterations, many questions remain regarding the mechanisms controlling IL-33 secretion. The current study strongly suggests that a HDM-induced increase in glycolytic flux facilitates IL-33 secretion. However, these data are not sufficient to pinpoint the exact pathways and processes regulating IL-33 release. Three main questions that remain to be answered are: what are the cytosolic compartments in which IL-33 resides? Which organelles participate in IL-33 translocation? What post-translational modifications are involved in IL-33 secretion?

As previously reviewed, IL-33 exhibits multi-compartmental localization inside cells [247]. Kakkar et al. reported that newly synthesized IL-33 was initially shuttled into the nucleus to associate with euchromatin, these molecules then utilized the nuclear pore complex to transit into the cytoplasmic space and reside in membrane-bound vesicles upon stress [247]. In our study, we confirmed that IL-33 was located in both the nucleus and cytosol, but we did not define the exact location

of IL-33 storage in the cytosol. Although our data implied that the IL-33 secreted from airway epithelial cells was derived from both nuclear and cytosolic compartments, direct evidence for the translocation is lacking. Considering these limitations and caveats, more detailed analyses of IL-33 intracellular localization (e.g. organelle(s) that stores IL-33, and organelle(s) that participates in IL-33 translocation after HDM exposure) are needed.

It is currently proposed that cytokines lacking a conventional signaling peptide, such as IL-33, may be secreted by pathways independent of ER-Golgi-mediated trafficking [440]. However, we were surprised to see that treatment of BEAS-2B cells with a specific inhibitor of vesicular traffic from the ER to the Golgi, Exo1 [423], also modulated HDM-induced release of IL-33 (Chapter 4). Normally, proteins are synthesized and assembled in the ER and then transported to the Golgi complex for further processing, sorting and packaging for secretion [482]. This process allows protein quality control, surface molecule export as well as post-translational modifications to happen. Obviously, this process could also play a role in HDM-induced IL-33 release. Since IL-33 release is independent of *de novo* protein synthesis (Chapter 3), other functions associated with the ER-Golgi network, for example, post-translational modifications (e.g. cleavage) of IL-33 or other molecules involved in its translocation, might be required for IL-33 release, For IL-33 itself, multiple lines of evidence have suggested that it undergoes post-translational modification prior to secretion [243, 252]. Specifically, it has recently been shown that IL-33 requires proteolytic processing by calpain for maturation [243], and that HDM-induced IL-33 release involves calpain-mediated processes [245]. These data

pose even more questions: is glycolysis-facilitated IL-33 release restricted to the mature form of the protein? Since ER and Golgi are also membrane structures that may preferentially utilize glycolytic ATP, does increased glycolysis also contribute to the post-translational modification of IL-33? Furthermore, are there any other signaling molecules that undergo post-translational modifications required for HDM-induced IL-33 release? Future studies are required to explore these remaining questions.

IL-33 was reported to undergo shuttling between cytosolic vesicles and the nucleus [247], probably because of its role in gene expression regulation. In fact, IL-33 was considered a dual functional protein with an intracellular role as a transcriptional regulator [483] distinct from its extracellular cytokine properties. As a transcriptional repressor, studies have suggested that nuclear IL-33 sequesters nuclear NF-κB and reduces NF-κB–triggered gene expression [484]. This new finding may shed light on another perspective of HDM-induced IL-33 as turning on a series of inflammation-promoting signals in airway epithelial cells and contribute to allergic inflammation. According to this, the inducible expression of *Il33* in immune cells such antigen presenting cells, mast cell and granulocytes could play regulatory roles that can be disturbed if IL-33 is released in response to external stimulation. In general, these data suggests that the release of IL-33 by various cells contributes to the development of allergic inflammation not only by its action as an extracellular cytokine, but also by activation of NF-κB and downstream signaling.

6.2.4 Validation and replication of the current discoveries.

We have identified a novel mechanism by which HDM induced glycolysis contributes to the live cell secretion of IL-33. Although our data strongly support this contention, future studies validating the current discoveries are needed. As discussed earlier, the specificity and effectiveness of certain inhibitors and siRNA used in this thesis remains to be proved. For example, Rac1-GTP activities and levels together with direct visualization of cytoskeleton rearrangement alterations should be tested respectively in cells treated with Rac1 inhibitors and siRNA. Intracellular calcium flux should be measured to reflect the effect of EDTA chelation, suramin inhibition of P2 purinergic receptors, as well as extracellular ATP. Analyses which allow us to visually track IL-33 translocation are needed in order to understand the effects we observed with ER-Golgi blockade. Furthermore, the successful blockade of AMPK signaling and TBK1 should be confirmed with the protein phosphorylation status and the protein levels.

In addition, inconsistencies between our results and recent published data suggest that differences exist between different cell lines. The results of the current study remain to be reproduced in primary normal and asthmatic epithelial cells. Moreover, the transformed human airway epithelial cell line (BEAS-2B) that we utilized in the current studies limits the generalizability of the observed phenotype to human disease as the normal human epithelial layer contains multiple cell types [485], in which inter-cellular communication between cells play a critical role in airway function. Given this, future studies are needed to confirm the role of altered cellular metabolism on IL-33 secretion in more cell culture systems which allow for cellular differentiation such as that observed in air-liquid interface cultures, *ex vivo*

culture of primary human airway epithelial cells from asthmatic and healthy donors, and/or mouse epithelial cells. Besides replication of the discoveries *in vitro*, the pathways identified (e.g., Rac1, AMPK, etc.) should be tested for their involvement in allergic asthma pathogenesis *in vivo* using murine models.

6.2.5 Future research for the role of HDM-induced glycolysis and IL-33 in allergic asthma *in vivo*.

Aside from the *in vitro* analysis of HDM-induced IL-33 release, our study using a murine model of experimental asthma provided strong evidence that HDM-induced early increases in glycolysis and IL-33 production contributed to the pathogenesis of allergic asthma *in vivo*. Blocking HDM-induced glycolysis abrogated allergic airway inflammation by inhibiting IL-33 recruitment of ILC2s and its priming of type-2 immune responses by IL-13. However, we were unable to provide direct evidence that the lactate and IL-33 were derived mainly from airway epithelial cells. To further understand this, *in vivo* radioactive labeling of glucose as well as pulse labeling of IL-33 may provide some information for the major source of lactate and IL-33.

In our study, DCA was given systemically to mice to inhibit glycolysis. This approach did not provide specific targeting of glycolysis in airway epithelial cells, and may complicate the interpretation of our data. Although we did show in the rIL-33 induced allergic airway response experiment that DCA did not exhibit effects on downstream responses to IL-33, we cannot rule out the possibility that DCA also targeted IL-33 producers other than airway epithelial cells. To specifically target

glycolysis in the airway epithelial cells, future studies using shRNA designed to express specifically in the airway epithelial cells to knock down PDK1 or LDH will moderately reduce glycolytic activity in these cells, and answer this question.

Moreover, even though we have observed that IL-33 was sufficient to induce allergic airway responses, and recent literature suggest that it is necessary for the HDM-induced asthmatic phenotype in mice [486], whether epithelial cell derived IL-33 is necessary in our model remains to be proven. In order to determine this, the allergic phenotype should be examined in mice conditionally lacking IL-33 in airway epithelial cells.

6.3 Implications for human disease

The current study identifies a novel mechanism by which HDM exposure induces IL-33 release and allergic asthma pathogenesis. As previously mentioned, the understanding of allergic asthma pathology has grown robustly over the last few years, but the initiation of the disease remains poorly identified. Accumulating evidence has suggested a dominant role for airway epithelial cells and their release of type-2 immune response promoting factors such as IL-33 upon exposure to environmental stimuli. However, although many studies of the role of IL-33 in allergic inflammation have been conducted, a lot of questions remain to be answered: (1) what are the environmental factors inducing IL-33? (2) How is IL-33 induced? (3) How to modulate the induction of IL-33?

We have provided answers to these questions. First, we have confirmed that HDM exposure induces rapid release of IL-33 from airway epithelial cells. HDM is

considered causal to allergic asthma pathogenesis [30, 473], and its allergenicity is associated with the mites themselves as well as ligands derived from mite-associated bacterial and fungal products [487]. Some mechanisms by which HDM induces allergic airway inflammation include cleavage of intercellular epithelial tight junctions by its protease component Der p 1 to allow allergen delivery to antigen-presenting cells [224], functional mimicry of Toll-like receptor complex protein by its component Der p 2 to confer intrinsic adjuvant activity [320], etc. Recent studies also identified that HDM sensitization was associated with *Il33* expression in the bronchial mucosa [488], and a murine model study of experimental asthma showed a significant role of IL-33 in HDM-induced allergic inflammation [489]. Another recent study also showed rapid induction of IL-33 release by HDM [245], and our study further confirmed this observation, highlighting the potential of targeting HDM-induced IL-33 release in patients.

Second, we have identified a novel mechanism for IL-33 release in which HDM induces the rapid increase in glycolytic flux thereby facilitating IL-33 release. As the induction and secretion mechanisms of IL-33 have garnered great interest during recent years, much has remained unknown about the exact pathways. Although studies have identified that IL-33 release is mediated by nuclear pore complex, cytoskeleton and calcium [244, 245, 247], no conclusion has been made to connect these sporadic evidence pieces together, and systemic elucidation of secretory mechanism of IL-33 from airway epithelial cells has been lacking. Here we attempt to provide some insights from the perspective of cell metabolism, and show that HDM-induced rapid increase of glycolysis facilitates the bioenergetics demand

of cells for IL-33 translocation by cytoskeleton. As mentioned above, our data not only suggests potential pathways for IL-33 release, but also confirmed the importance of cell metabolism in allergic diseases. In fact, the significance of cell metabolism in the field of immunology is gradually being appreciated. Recent studies have shown that alterations of cellular metabolism direct essential pathways in immunology such as dendritic cell activation [338], macrophage polarization [490], and T cell differentiation [341]. Our study extended the knowledge of cell metabolism in health and disease to airway epithelial cells, adding to the current understanding of their important roles in immune response and allergic asthma pathogenesis.

Most importantly, we showed that modulating cellular glucose metabolism was also capable of modulating IL-33 release *in vivo*, leading to reduced allergic airway responses via inhibition of ILC2 recruitment and its production of IL-13. The reduction in all aspects of allergic airway inflammation suggested blockade of disease initiation. This observation provides a potentially new avenue for the treatment of asthma attacks. The inhibitor utilized in the current studies, DCA, has been also used for clinical treatment of lactic acidosis [394, 395] and cancer [398], indicating its great potential for future clinical application in allergic asthma intervention. It is also worth noting that in our studies, DCA is mainly given during the initiation phases of allergic airway inflammation. In another study in which DCA was delivered throughout the allergen sensitization and challenge periods, DCA also reduced ragweed-induced allergic airway inflammation by inhibition of CD4+ T cell activities [370]. Although further studies detailing the optimal regimen of DCA

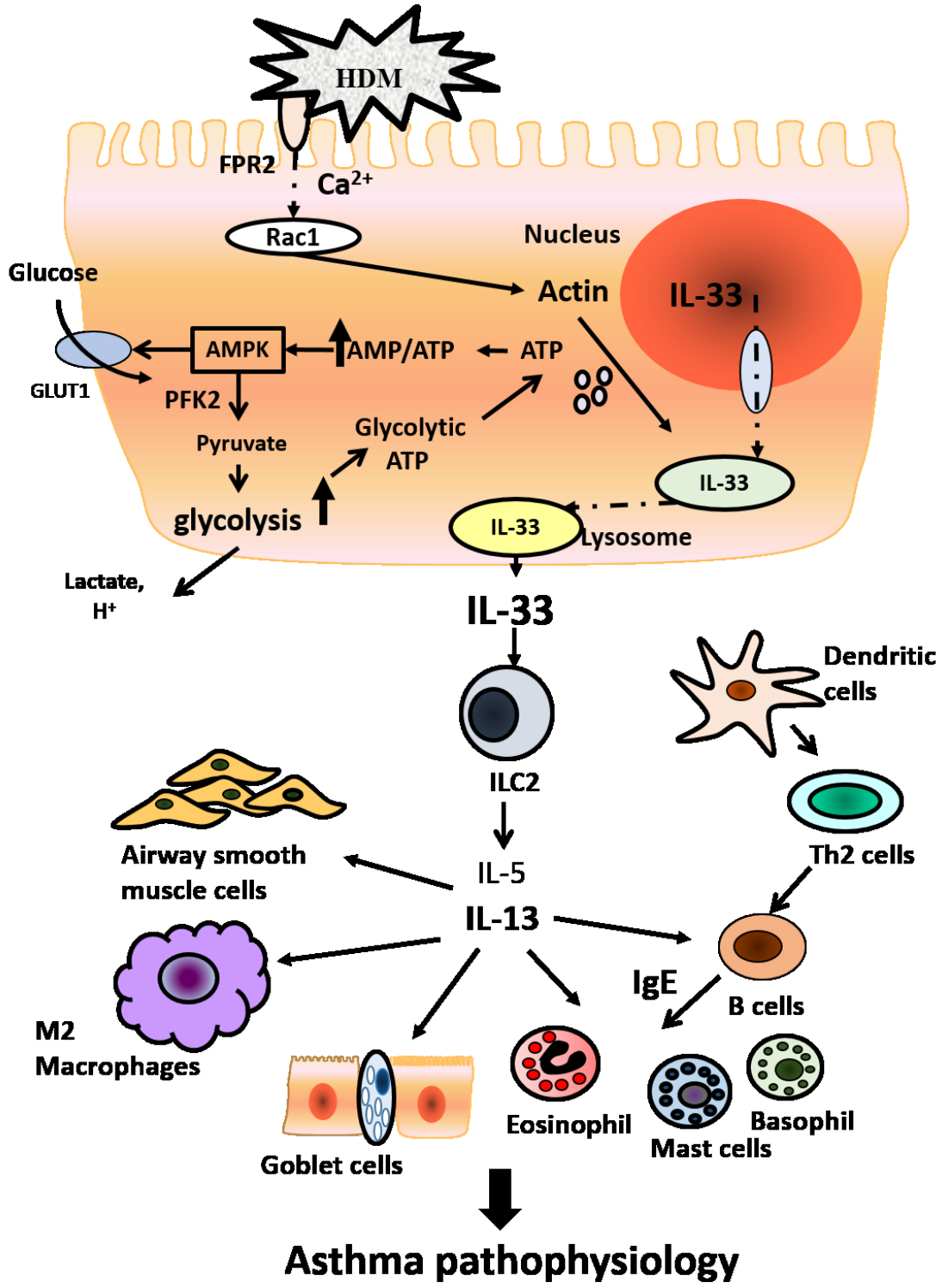
administration and its modes of action *in vivo* are still needed, our study suggest that DCA may be a potentially promising new therapeutic approach for the treatment of asthma.

Our findings have implications beyond the context of allergic asthma. IL-33 has been shown to play important roles in many diseases and conditions such as obesity, intestinal inflammation, and cancer [491], in which cellular metabolism may also play a role. Moreover, the pathways reported herein may apply to structural cell secretion of IL-33 in other organs. As such, the mechanisms identified in our model could be explored in other diseases and conditions in which IL-33 has been shown to play a role, and could be potentially exploited in cases where modulating IL-33 release could affect disease outcomes.

6.4 Figures

Figure 20: Schematic summary of epithelial cell metabolism in allergic asthma pathogenesis. HDM exposure activates FPR2 that is expressed by airway epithelial cells. FPR2 activation results in rapid mobilization of intracellular Ca2+ and rapid cytoskeleton rearrangements mediated by Rac1 to facilitate translocation and release of IL-33. This process rapidly consumed ATP presented in the cytosol, leading to altered AMP/ATP ratio and activation of AMPK signaling. The AMPK then led to an increase of glycolysis probably by promoting glucose uptake and glycolytic enzyme activities. This rapid increase of glycolysis then generates glycolytic ATP to facilitate the early release of IL-33. IL-33 recruits and activates ILC2s, the major innate producer of IL-13. IL-13 primes type-2 immune responses and contributes to airway hyperresponsiveness.

Figure 20: Schematic summary of epithelial cell metabolism in allergic asthma pathogenesis.



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beyond Th2 immunity-emerging roles in obesity, intestinal inflammation, and cancer. Eur J Immunol, 2016. **46**(5): p. 1091-100.

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Publications

- Li M,* Luo XJ,* **Xiao X**,* Shi L, Liu XY, Yin LD, Ma XY, Yang SY, Pu XF, Yu J, Diao HB, Shi H, Su B.† Analysis of common genetic variants identifies RELN as a risk gene for schizophrenia in Chinese population. ***The World Journal of Biological Psychiatry*** 2013; 14:91-9.

- Luo XJ,* Li M,* Nho K, Deng M, Chen Q, Weinberger DR, Vasquez AA, Rijpkema M, Mattay VS, Saykin AJ, Shen L, Fernández G, Franke B, Chen JC, Chen XN, Wang JK, **Xiao X**, Qi XB, Xiang K, Peng YM, Cao XY, Li Y, Shi XD, the Alzheimer's Disease Neuroimaging Initiative, Gan L,† Su B.† The interleukin 3 gene (IL3) contributes to human brain volume variation by regulating proliferation and survival of neural progenitors. ***PLOS ONE*** 2012; 7:e50375.

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Posters and Presentations

- Xiao X, Lajoie S, Afzal J, Breysse P, Wills-Karp M, (Poster Presentation)

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Honors and Awards

2008	1st Prize, Outstanding Scientific Research Achievements by Students of Wuhan University
2008	Outstanding Scientific Research and Innovative Experiment by Students of Wuhan University
2006 – 2008	Excellent Undergraduate Scholarship
2006	2nd Prize, Wuhan University's Outstanding Achievements for Summer Practice

Skills

Experimental Skills

- Common murine study procedures (intra peritoneal injection, intro nasal and intra tracheal challenge), dissection, and major organ harvesting.
- Murine bone marrow cell isolation and differentiation.
- Single cell suspension and flow-cytometry analysis.
- Enzyme Linked Immunosorbent Assay
- DNA extraction, PCR, Genotyping.
- RNA isolation, purification and quantitative real-time PCR.
- Vector construction and molecular cloning techniques.
- Regular cell culturing, cell transfection and dual-luciferase reporter assay system.
- Cell apoptosis and viability assays.
- Cell respiration measurement.
- Western Blot.
- Data analysis using R software.

Professional skills

- Familiar with emerging issues in the workplace such as workers' compensation, disability, employee assistance programs, etc.
- Understanding of basic management thoughts associated with occupational health, safety, and environmental management.
- Managed common methods in safety and occupational health management including loss tree analysis, root cause analysis, One Point Lessons, and visual preventive methods.
- Create and document exposure assessment
- Critique risk analyses
- Familiar with tools of risk management including laws, regulations, public health guidance, and community communication.